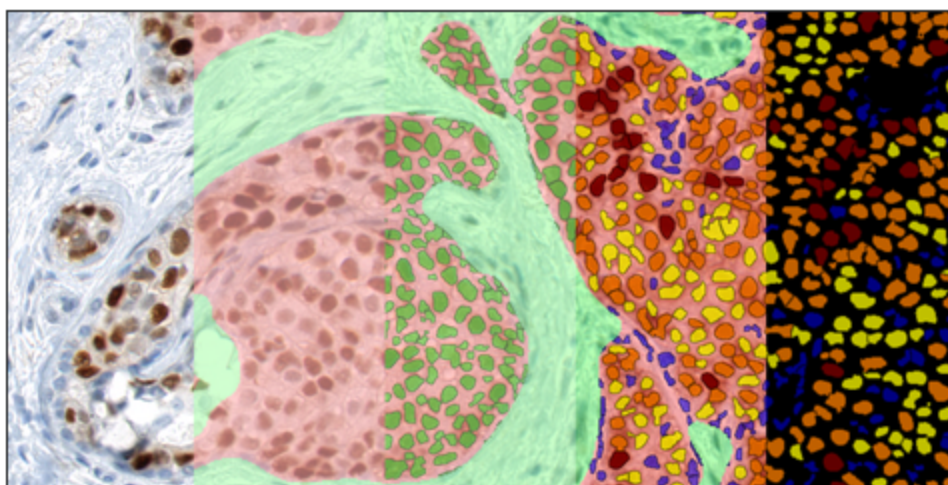


inForm[®] V2.0.2

User's Manual



inForm[®] 2.0 – Tissue Finder
Advanced Image Analysis Software

US Patents 7,555,155; 7,953,264; 8,280,140 and patents pending.
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This manual describes how to use inForm version 2.0.2 software.

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This software covered by US Patent 7,555,155; 7,953,264; 8,280,140; and patents pending.

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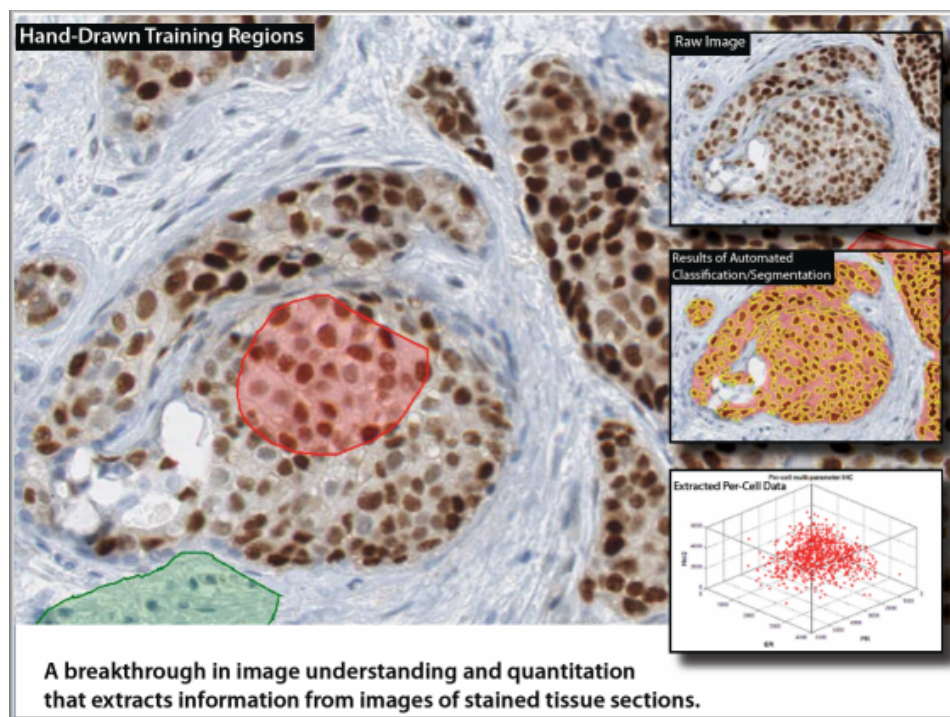
1 Welcome to inForm

PerkinElmer's inForm® software has been developed to address the biggest challenge facing microscopists once quantitative imagery has been acquired: extracting data. The challenge increases when dealing with multiplexed assays, since signals can be weak and highly mixed. inForm provides a powerful and fast solution with an intuitive, easy-to-use software interface. What might have been impossible or have taken days, can now be done in minutes.

inForm is an advanced image analysis package that solves challenging image analysis problems by combining fast and easy learn-by-example automated image processing with object recognition and data analysis tools. It is based on machine learning, which means that you can train the program to create an effective solution by simply drawing around examples of what you want segmented within the images. inForm then creates an algorithm that you can subsequently apply across the entire image or across as many images as you want.

PerkinElmer's multispectral images and industry-standard RGB images, including TIFF files generated using PerkinElmer's Slide Imaging systems, can be read and analyzed by inForm.

inForm is for research use only.



Getting started – new users

- Be sure to review the topics in the [Introduction](#) ⁷ to familiarize yourself with the features and specifications of the software.

2 Introduction

The topics in this section provide basic information about inForm, what it is for, and what you can do with it.

2.1 About inForm

inForm operates on monochrome, color, or multispectral images of tissue sections. Sections can be stained with standard stains, immunohistochemical stains (IHC), and immunofluorescence stains (IF, including Qdots™). Stains can be single or multiplexed for multi-analyte analyses to support applications such as signaling pathway research in oncology.

inForm is available in different configurations, each providing additional capabilities for different applications.

- **inForm Tissue Finder** can be trained to find virtually any tissue type or structure, such as tumor, fibrosis, inflammation, stroma, granuloma or vessels.
- **inForm Cell Analysis** can be used to assess IHC or IF staining levels, on a cell-by-cell basis, for cell prototyping. It can also generate object counts.
- **inForm Basic Analysis** can generate area statistics and quantitative stain levels for hand-drawn or threshold-generated regions.
- **inForm Viewer** can visualize component data, and reconstruct the unmixed signals into a composite image.

— Features

- Compatibility with PerkinElmer's multispectral images (.im3 and .im4) and with conventional color images (.tif, .jpg, etc.).
- Full range of software configurations which can include capabilities from simple tissue area measurements to cell-as-a-unit multiplexed molecular phenotyping. To view the levels of inForm and the capabilities of each level, see [Software Configurations](#)⁸.
- Computationally efficient; runs on a standard laptop.

— Specifications

- Operates on PerkinElmer's multispectral images (.im3 and .im4), and monochrome or color images (TIFF, BMP, JPEG, PNG)
- Computer Specifications: Microsoft® Windows® 7, 64-bit with 8GB RAM recommended. (Minimum: Microsoft® Windows® 7, 32-bit with 2GB RAM)

2.2 Software Configurations

inForm is available in four different software configurations. Each configuration enables specific tasks for viewing, selecting, segmenting, and counting.

The following inForm software configurations are available:

Viewer

inForm Viewer includes the following features:

- View images
- Unmix images
- View components and their signals
- View a composite of the component images
- Export images

Basic Analysis

inForm Basic Analysis includes all of the features available in inForm Viewer, plus the following features:

- Manually segment tissue
- Create threshold maps
- Create colocalization maps
- Generate quantitative data for threshold and colocalization maps
- Save projects and algorithms
- Batch process images
- Export data tables

Cell Analysis

inForm Cell Analysis includes all of the features available in inForm Basic Analysis, plus the following features:

- Use cell segmentation to identify cells and cellular compartments
- Score segmented cells
- Use object segmentation to identify objects

Tissue Finder

inForm Tissue Finder includes all of the features available in inForm Cell Analysis, plus the following features:

- Train the Tissue Segmenter to automatically identify tissue categories
- Automatically segment the image into tissue categories
- Merge data files from a set of images or slides into summary data files

Steps Available in Each Software Configuration

The table below specifies which algorithm steps are available in each software configuration.

	Viewer	Basic Analysis	Cell Analysis	Tissue Finder
Prepare Images	X	X	X	X
Manual Tissue Segmentation		X	X	X
Trainable Tissue Segmentation				X
Threshold		X	X	X
Colocalization		X	X	X
Cell Segmentation			X	X
Object Segmentation			X	X
Score (only used with Cell Segmentation)			X	X
Export	X	X	X	X

Batch Processing is available with inForm Basic Analysis or higher.

Review/Merge is available with inForm Tissue Finder.

2.3 Key Terms

The following terms describe components of inForm. You should understand the terms below when using the software.

Algorithm	Algorithms include all steps in processing an image, from image preparation, tissue segmentation and/or cell segmentation, object counting or scoring, to export. Algorithms may also include threshold or colocalization settings, depending on the project created to analyze the images.
Processing Region	Processing regions are user-drawn regions of interest. Use processing regions to only process specific regions on an image. All areas outside of the marked processing regions are ignored.
Processing Set	A set of images that will be processed using the settings in the Manual Analysis tab. Images in the processing set display a green square on the image thumbnail when viewing images in Single mode. All images added to a project are included in the processing set by default. To add or remove images from the processing set, right-click on the large image in the image display area and click Processing Set. You can also click the Image List Editor button to open the Available Images window and then click the Load Images button.
Project	inForm projects include the images that were added to the project and the settings for each step in the algorithm. A new project that contains only the Prepare Images and Export Step is automatically created when opening inForm. You can work in the new default project, create a new project, or open a saved project.
Tissue Category	Tissue Categories identify structures such as tumor cells vs. healthy cells that are present in the tissue samples. Regions in the image can be marked manually using inForm Basic Analysis or higher. Use inForm Tissue Finder to use the Tissue Segmenter to automatically classify tissue.
Tissue Segmenter	The Tissue Segmenter automatically identifies regions of tissue or regions that correspond to specific types of tissue. When you “train” a tissue segmenter, it learns how to identify structures in the images based on the training regions within each tissue category. The tissue regions or areas detected with the tissue segmenter can then be segmented to detect cells and perform IHC or IF scoring, or can be used for object counting. Only available with inForm Tissue Finder.
Training Set	The set of images that is used to train the Tissue Segmenter to identify tissue categories automatically. Images are automatically added to the training set if a training region is drawn on the image. To remove an image from the training set, right-click on the large image in the image display area and click to deselect Training Set. When an image is removed from the training set, the training regions on the image are not used when training the Tissue Segmenter. (The training regions are not deleted from the image, so the image can be added back to the training set if desired.) You can also use the Available Images window to add or remove images from the training set.

2.4 Using Online Documentation

This manual contains detailed information about the inForm application. It is designed to be used as a reference tool in your everyday work with inForm. The manual explains how to use inForm through detailed explanations of features and step-by-step procedures for common tasks using realistic scenarios. The PDF file can be read online or can be printed for reference.

inForm also includes complete documentation in an HTML-based help system, which includes all of the information in this User's Manual. Online help provides three ways of locating information: Use the Contents tab to navigate through the document, the Index tab to find topics by keyword, or the Search tab to look up topics that contain specific words or phrases.

2.5 Installing and Starting inForm

Close all applications that are running before beginning the inForm installation. Installation requires an administrator account.

Starting the Install from CD, DVD, or USB Drive:

Insert the inForm CD, DVD, or USB drive. If AutoRun is turned on, the installation wizard starts automatically. If the installation does not start automatically, in Windows Explorer, navigate to the install media and double-click inFormSetup.exe.

Starting the Install from a Network or Downloading:

If you are installing inForm from a network location, download and extract the inForm installation folder to a local drive. Double-click on inFormSetup.exe to start the installation.

Installing the Software:

Follow the wizard prompts to install the software. inForm requires the Microsoft .NET 4.5 framework. The installer will install Microsoft .NET 4.5 if it is not already installed. The installer asks if you will be using inForm to create Vectra[®] procedures to analyze Vectra data. Be sure to choose **Yes** if you want to be able to create algorithms to use with the Vectra software.

Starting inForm:

Select **Start > All Programs > PerkinElmer > inForm > inForm 2.0.2**,
OR
double-click the **inForm** shortcut on the Windows desktop.

To use any software configuration other than inForm Viewer, see [Activating the License](#)¹².

2.6 Activating the License

After installing inForm, only the options for inForm Viewer are available. The customer license must be activated to enable any purchased software options. License activation requires an administrator account.

To activate a customer license:

1. Log into the computer using a Windows Administrator account.
2. Start inForm.
3. Select **License** → **Activate** or click the **Activate** button. The Activation window opens.
4. Enter your 20 character license number.
5. If available, use the online activation method to complete your activation.
6. If online activation is not available, click the **Copy to Clipboard** button to copy the text onto the Windows clipboard.
7. Email the copied text to the email address shown in the Activation window.
8. Click the **Cancel** button to close the Activation window.
9. Wait to receive the email that contains the text for the response key.
10. Start inForm and select **License** → **Activate**. The Activation Window opens.
11. Type or paste the response key from the email into the Activation window and click the OK button. inForm displays a restart message.
12. Click the **OK** button. inForm restarts automatically. The purchased options are now available in the software.

Transferring a Software License

You can transfer the license from the current computer to another computer. When the license is transferred to the new computer, the license is removed from the current computer, leaving only the inForm Viewer options.

Transferring a license requires an administrator account on both machines, a USB thumb drive, and an available USB port on each machine.

To transfer the license, log into the unlicensed computer with an administrator user name and password. Start inForm and select **License** → **Activate**. Choose **Transfer**. Follow the instructions provided in the dialog boxes to complete the transfer.

2.7 Contacting PerkinElmer

For more information, contact PerkinElmer or your local authorized PerkinElmer distributor:

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PerkinElmer Web Site:

 <http://www.PerkinElmer.com>

Technical Support Email:

 global.techsupport@perkinelmer.com

3 Understanding the inForm Work Area

The inForm work area contains the step bar, process editor panel, image display toolbar, and image display area. The example below shows the Manual Analysis tab with a brightfield image that has been segmented into two tissue categories.

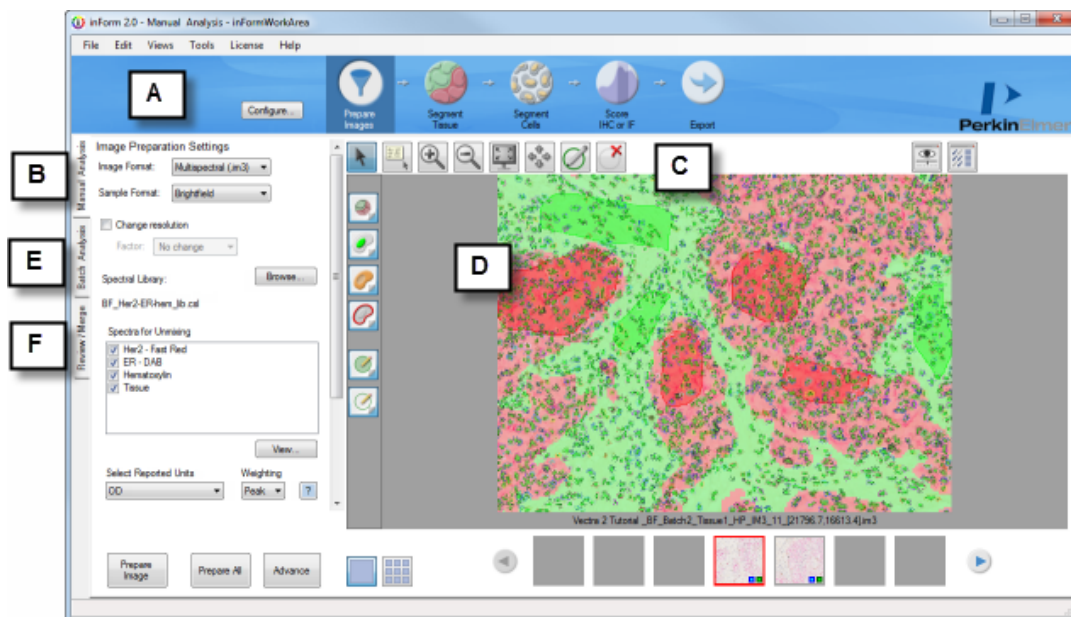
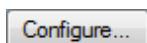


Figure 1. inForm Work Area with Image Showing Segmented Tissue

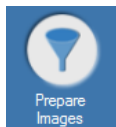
— A. Step Bar

The Step bar displays an icon for each processing step included in the project that is open in the Manual Analysis tab. The Step bar only displays when the Manual Analysis tab is selected. A new project is automatically created when inForm opens. By default, the new project only includes the Prepare Images and Export steps. To create a new project with additional steps, see [Creating Projects](#)^[27]. To add, remove, or change the steps in the open project, see [Configure a Project](#)^[28].

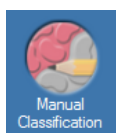
Click the icons in the step bar at the top of the work area to display the settings for each step in the Manual Analysis tab. For example, click **Prepare Images** to display the Image Preparation Settings panel in the Manual Analysis tab; click **Export** to display the Export Settings panel.



Configure button - Opens the Configure Project window to change the steps in the current project. See [Creating Projects](#)^[27] for instructions. Vectra[®] Tissue Finder and Vectra HPF Finder projects cannot be configured.



Prepare Images - Use to specify the image format and sample format, change the image resolution, load a spectral library, convert images to optical density, and unmix images. See [Preparing Images](#)^[35] for instructions.

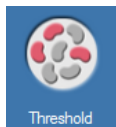


Manual Classification - Use to create tissue categories and manually draw tissue regions on the images in the project. Exported data includes position, shape, and component signal statistics for each segmented tissue region. See [Manual Tissue Segmentation](#)^[44] for instructions. This feature is available with inForm Basic Analysis

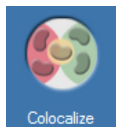
or higher.



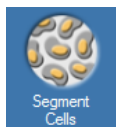
Segment Tissue - Use to train the automated tissue segmenter and automatically segment the images. Exported data includes position, shape, and component signal statistics for each segmented tissue region. See [Trainable Tissue Segmentation](#)^[47] for instructions. This feature is only available with inForm Tissue Finder.



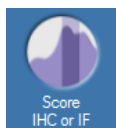
Threshold - Use to create and analyze threshold maps. Exported data includes position, shape, and component signal statistics for each thresholded region. See [Threshold](#)^[54] for instructions. This feature is available with inForm Basic Analysis or higher.



Colocalize - Use to create and analyze colocalization maps. Exported data includes position, shape, and component signal statistics for the colocalization region, as well as position, shape, and component signal statistics for the underlying component regions. See [Colocalization](#)^[57] for instructions. This feature is available with inForm Basic Analysis or higher.



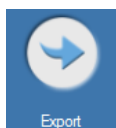
Segment Cells - Use to segment cell nuclei and optionally segment cytoplasm and membrane. Exported data includes position, shape, and component signal statistics for each segmented cell. See [Segmenting Cells](#)^[59] for instructions. This feature is available with inForm Cell Analysis or higher.



Score IHC or IF - Use to quantify IHC or IF staining levels in segmented cells. The tools provided are intended to automate visual assessment (Positivity, 0-3+, 10-bin, 50-bin, and Double Positivity) and to provide histogram data of staining levels. Exported data includes the score for each selected cell compartment. See [Scoring IHC or IF](#)^[66] for instructions. This feature is available with inForm Cell Analysis or higher.



Count Objects - Use to segment objects other than cells. Exported data includes the position, shape, and component signal statistics for each segmented object. See [Counting Objects](#)^[71] for instructions. This feature is available with inForm Cell Analysis or higher.



Export - Use to select the export directory, the images to export, the data to export, and the file name options. See [Exporting the Data](#)^[89] for instructions.

B. Manual Analysis Tab

The **Manual Analysis** tab on the left displays the settings for each step. Use these panels to select the settings for the algorithm or project. These settings can only be edited when viewing a single image, and not while viewing images in Gallery mode. See also [Image Display Area](#)^[18].

C. Toolbar

The toolbar enables you to adjust the image zoom level, draw processing regions, draw training regions, and adjust the view settings. The tools available on the toolbar change based on the current processing step. The toolbar displays in the Manual Analysis tab.



Select: Return the cursor to the default pointer mode.



View Component Data: Hover over the image to display the signal intensity of each component at any location.



Zoom In: Click on the image to re-center the image and to zoom in. Click and drag to zoom in on a specific region of the image.



Zoom Out: Click on the image to re-center the image and to zoom out. Double-click the image with this tool to return magnification to 100%.



Zoom to Full: Click to view the image at 100%.



Pan: Click and drag the image to view the desired region in the window.



Draw Processing Regions: Click and drag to outline the desired processing region. Only the areas of the image within the processing regions are analyzed.



Delete a Region: Click inside a processing or training region to remove the region.



Draw Training Regions: Click and drag to identify regions of the image that represent the selected tissue category when training the tissue segmenter. This tool selects automatically when you select a tissue category from the category list. When in draw mode, you can continue drawing training regions. Only available during the **Segment Tissue** step.



Draw Classification Regions: Click and drag to identify regions of the image that contain the selected tissue category. Only available during the **Manual Classification** step.



Select Regions to Unclassify: Click and drag to outline the area on the image to unclassify. Only available during the **Manual Classification** step.



Select Pixels to Unclassify: Click and drag over the specific pixels to remove from the classification region. Only available during the **Manual Classification** step.



White Picker: Click on a white area on a Brightfield image to select the white background spectra prior to converting the image to OD (optical density). Available only

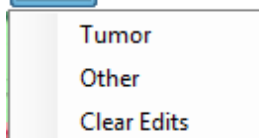
during the **Prepare Images** step.



Add to Thresholded Regions: Click the button to select the desired unmixed component and then click and drag to outline the area on the image to be added to the selected threshold map. Only available during the **Threshold** step.



Erase Thresholded Regions: Click the button to select the desired unmixed component and then click and drag to outline the region to remove from the threshold map. Only available during the **Threshold** step.



Edit the Tissue Segmentation: Click the button to select the tissue category and then click and drag to manually draw the selected tissue category over an area. After segmenting the images, inspect the images to make sure the tissue regions are segmented as you intended. If areas are under the wrong masks (the tumor mask over non-tumor cells, for example), use this tool to draw the desired mask over an area. Only available during the **Segment Tissue** step. Select Clear Edits to clear all manual edits to the tissue masks.



View Editor: Click to open the [View Editor Window](#)¹⁹ to choose the type of image or table to view and to change the display settings. You can also view or hide cells, masks, and regions. Buttons to quickly show or hide image maps are available on the left side of the image display area.



Image List Editor: Click to opens the [Available Images Window](#)²¹ to add images to or remove images from the open project, the processing set, or the training set.

Show/Hide Map Options: Click to show or hide the image maps. You can show or hide segmentation maps, training regions, and processing regions. (The Show/Hide options are also available on the [View Editor](#)¹⁹.) The buttons include:



Tissue Segmentation Map - Click to show/hide the Tissue Segmentation Map.



Nuclear Segmentation Map - Click to show/hide the Nuclear Segmentation Map.



Cytoplasm Segmentation Map - Click to show/hide the Cytoplasm Segmentation Map.



Membrane Segmentation Map - Click to show/hide the Membrane Segmentation Map.



Training Regions - Click to show/hide the Training Regions.



Processing Regions - Click to show/hide the Processing Regions.

D. Image Display Area



The image display area in the Manual Analysis tab has two modes -- *Single* and *Gallery*.

- **Single mode** displays one image in the large Image area, and displays a row of thumbnails of all other open images below the large image. Click any thumbnail to view the image in the display area. A blue square on a thumbnail indicates that the image is included in the training set; a green square indicates the image is in the processing set. The Algorithm settings in the left-hand panel can only be edited in *Single* mode.
- **Gallery mode** displays all the project images in the large Image area. Use the Image Size slider at the top of the workspace to adjust the number of open images shown in the image display area. In *Gallery* mode, all Algorithm settings are read-only. *Gallery* mode is for image and table review only; you cannot edit the Algorithm settings in this mode.

Right-Click Shortcut Menu

The following actions are accessible by right-clicking on an image in the Image Display area:

- **Save:** Saves a copy of the image (as shown in the Image Display Area) to a file in the selected format (.tif, .jpg, .png, .gif, or .bmp). (Default is the last selected image format.)
- **Delete Region:** Deletes the region that you right-clicked on. (Only available if you right-clicked on a region.)
- **Delete Regions:** Provides options to delete all training regions, all processing regions, or both, from an image. (Only available if training regions and/or processing regions have been created on the image.)
- **Show Regions:** Provides options to show/hide all training regions, processing regions, or both, on an image.
- **Processing Set:** Adds or removes the image from the processing set. If checked, the image is included in the processing set. If cleared, the image is not included in the processing set. Click on the option to toggle the check mark.
- **Training Set:** Adds or removes the image from the training set. If checked, the image is included in the training set. If cleared, the image is not included in the training set. Click on the option to toggle the check mark. Only displays if training images have been drawn on the image. (Draw training regions on the image or use the [Available Images Window](#) ² to add images without training regions to the training set.)
- **View Editor:** Opens the [View Editor](#) ¹⁹ to select the display options.
- **Remove from Project:** Removes the image from the project.

E. Batch Analysis Tab

After a suitable project or algorithm for processing images has been created, validated, and saved, use the Batch Analysis tab to process large numbers of images. Use the [Batch Analysis Editor](#)^[97] to select a project or an algorithm for batch processing, select export directory/folder preferences, and select the image/table options. (This feature is available with inForm Basic Analysis or higher.)

F. Review/Merge Tab

After completing a batch run, which often generates large numbers of output files, use the [Review/Merge Editor](#)^[93] to combine the data from multiple output files into a single file for detailed and quantitative analysis. The [Review/Merge Editor](#)^[93] has tools to filter out data from samples that are inadequately prepared, stained, and/or segmented, so that merged data sets are reliable. (This feature is only available with inForm Tissue Finder.)

3.1 View Editor

Use the View Editor to select the display options for images or data tables after processing. The images and data tables available are determined by the steps in the project. The steps available are determined by the inForm license. This section lists the views that are available for each software configuration. For definitions of the options, see [Displaying the Extracted Data](#)^[73]. To open the View Editor, click the **View Editor button** on the image toolbar.

Views Available In Viewer

Color Image: Shows the original color image plus the regions selected in the Image Options.

Component Image: Shows an image of a single unmixed component.

Composite Image: Shows a reconstructed image of all components.

Views Available In Basic Analysis

Includes all views in Viewer, plus:

Tissue Segmentation Data: This table is available when an algorithm contains a manual segmentation step. The table provides information on the manually segmented tissue regions. (See [Viewing the Tissue Segmentation Data Table](#)^[78] for detailed information.)

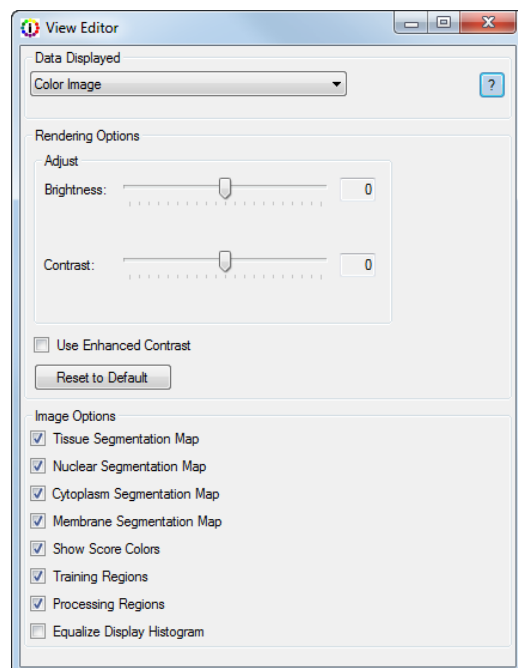


Figure 2. View Editor

Quant Data: This table is available when an algorithm contains a threshold or colocalization step. The table provides information on the thresholded regions. (See [Viewing the Quant Data](#)^[86] for detailed information.)

Colocalization Data: These tables are available when an algorithm contains a colocalization step. (See [Viewing the Colocalization Data](#)^[84] for detailed information.) There are three tables:

- **Colocalization Table:** Shows colocalization data of the selected components and positivity data for each selected component.
- **In Channel Table:** Shows the positivity of one component within another for all selected colocalization components.
- **Quant Table:** Shows stats for the whole image and colocalization region.

Views Available In Cell Analysis

Includes all views in Basic Analysis plus:

Cell Segmentation Data: This table is available when an algorithm contains a cell segmentation step. The table provides information on the segmented cells. (See [Viewing the Cell Segmentation Data](#)^[80] for detailed information.)

Object Segmentation Data: This table is available when an algorithm contains an object segmentation step. The table provides information on the segmented objects. (See [Viewing the Count Data](#)^[85] for detailed information.)

Score Data: This table is available when an algorithm contains a score step. The available data changes depending on the type of scoring (positivity, 0-3+, 10-bin, 50-bin, and Double Positivity). (See [Viewing the Score Data](#)^[82] for detailed information.)

Views Available In Tissue Finder

Includes all views in Cell Analysis. Tissue Segmentation data displays in the Tissue Segmentation Table. (See [Viewing the Tissue Segmentation Data](#)^[78] for detailed information.)

3.2 Available Images Window

Use the Available Images Window to view a list of all images in the project and to add images to or remove images from the processing set or the training set. (The training set is only supported in inForm Tissue Finder when training the Tissue Segmenter.) Click the **Image List Editor** button to open the Available Images window.



To load additional images into the project, click the **Load Image** button. The new images must be compatible with the Prepare Images settings in the current project. New images must have the same spectral wavelength characteristics as the existing images in the project.

To remove an image, right-click on the image name and click Remove.

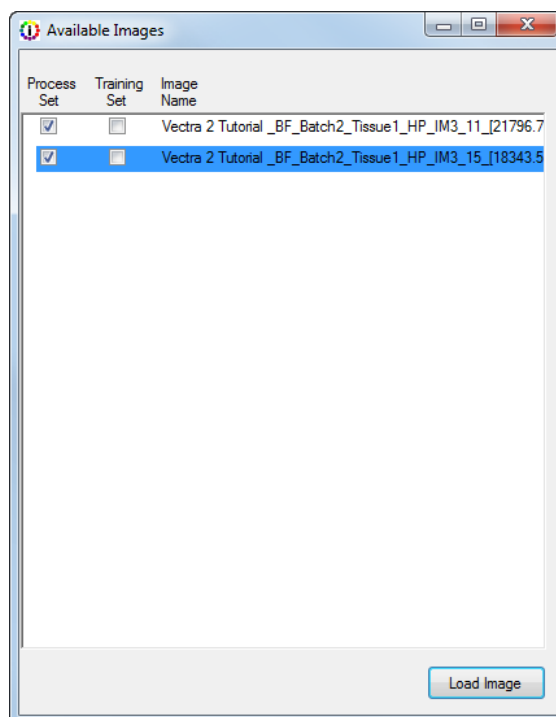


Figure 3. Available Images Window

3.3 New Project Window

Use the New Project Window to select the steps to include in the project. Certain steps are only available if a software option has been purchased and installed. (For available software configurations, see [Software Configurations](#)^[87]).

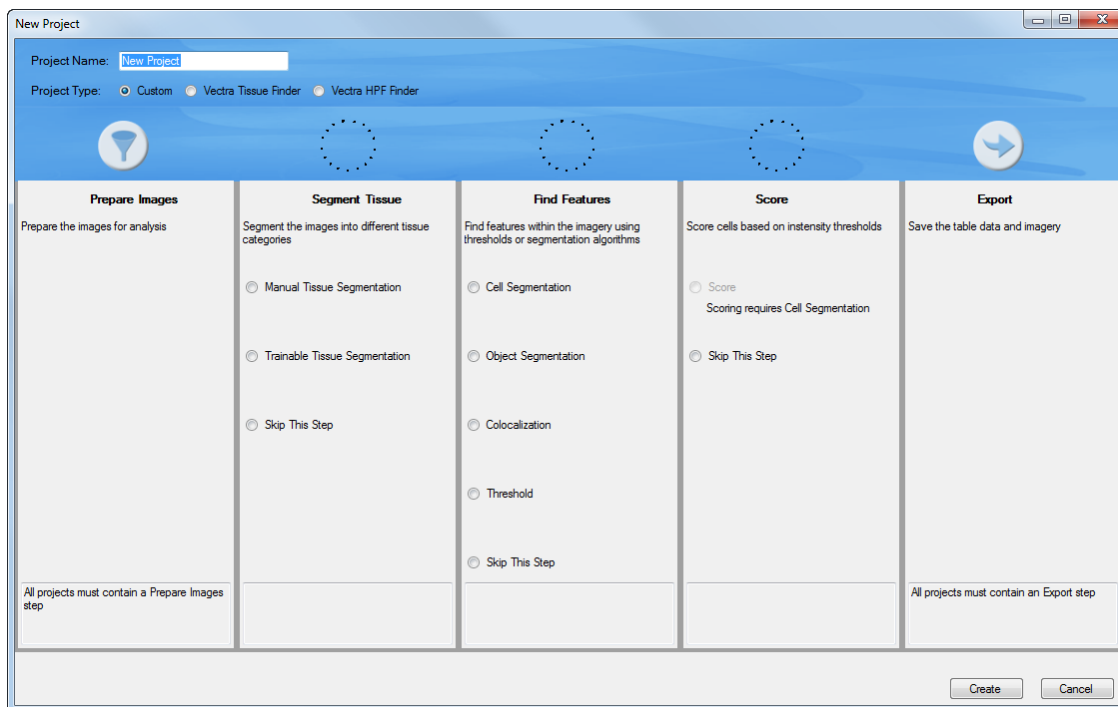


Figure 4. New Project Window

The New Project window contains the following options and buttons:

Project Type - These options only display in inForm Tissue Finder if you chose to enable Vectra algorithms when inForm was installed. If these options are not displayed, all new projects are Custom Projects.

- **Custom Project** option - Creates an inForm project with the selected steps.
- **Vectra Tissue Finder Algorithm** - Creates an algorithm to use in the Vectra software to identify areas of tissue. See [Creating Algorithms for Vectra](#)^[94] for details.
- **Vectra HPF Finder Algorithm** - Creates an algorithm to use in the Vectra software to identify areas of tissue where images should be taken using the high power objective. See [Creating Algorithms for Vectra](#)^[94] for details.

Prepare Images - Required for all projects. Specifies the Image Format and Sample format, changes the image resolution, loads a spectral library, unmixes the spectral components, and removes haze from the images. See [Preparing Images](#)^[35] for details.

Segment Tissue - Segment the image into specific tissue categories.

- **Manual Tissue Segmentation** - Tissue categories are manually drawn on each image. See [Manual Tissue Segmentation](#)^[44] for details.
- **Trainable Tissue Segmentation** - The tissue segmenter is trained to identify different tissue types in the images. Processing the images automatically assigns the appropriate tissue

categories. See [Trainable Tissue Segmentation](#)^[47] for details.

Find Features - Find features in the images using thresholds or segmentation algorithms.

- **Cell Segmentation** - Identifies individual cells or subcellular objects in the image using either thresholded or algorithmic segmentation. See [Segmenting Cells](#)^[59] for details.
- **Object Segmentation** - Identifies objects on an image using either thresholded or algorithmic segmentation. See [Counting Objects](#)^[71] for details.
- **Colocalization** - Identifies regions on an image where multiple unmixed component thresholds overlap. Threshold values for each component are specified individually. See [Colocalization](#)^[57] for details.
- **Threshold** - Identifies regions on an image where a component is above a specific threshold. See [Threshold](#)^[54] for details.

Score - Score cells based on intensity thresholds.

- **Scoring** - Scores the segmented nuclei, cytoplasm, and/or membrane to count the percent positive of a specific component above a specified threshold. See [Scoring IHC or IF](#)^[66] for details.

Export - Required for all projects. Specifies the export settings for images and data tables after processing is complete. See [Exporting the Data](#)^[89] for details.

3.4 Spectral Library Window

Use the Spectral Library window to view charts of the emission spectral curve for each stain selected for unmixing. Viewing the spectral library can help when troubleshooting unexpected unmixing results. Ideally, the spectra should be easily distinguishable in each of the imaging bands. Typically, one filter is used for each stain. If the Spectral Library uses multiple filters, one chart displays for each filter. The figure below shows a spectral library with DAPI, CY3, and CY5 with good separation in each chart. The Spectral Library window updates dynamically if the Image Preparation Settings are changed.

To open this window, click the **View** button under the Spectral Library list box in the Prepare Images step after selecting a spectral library.

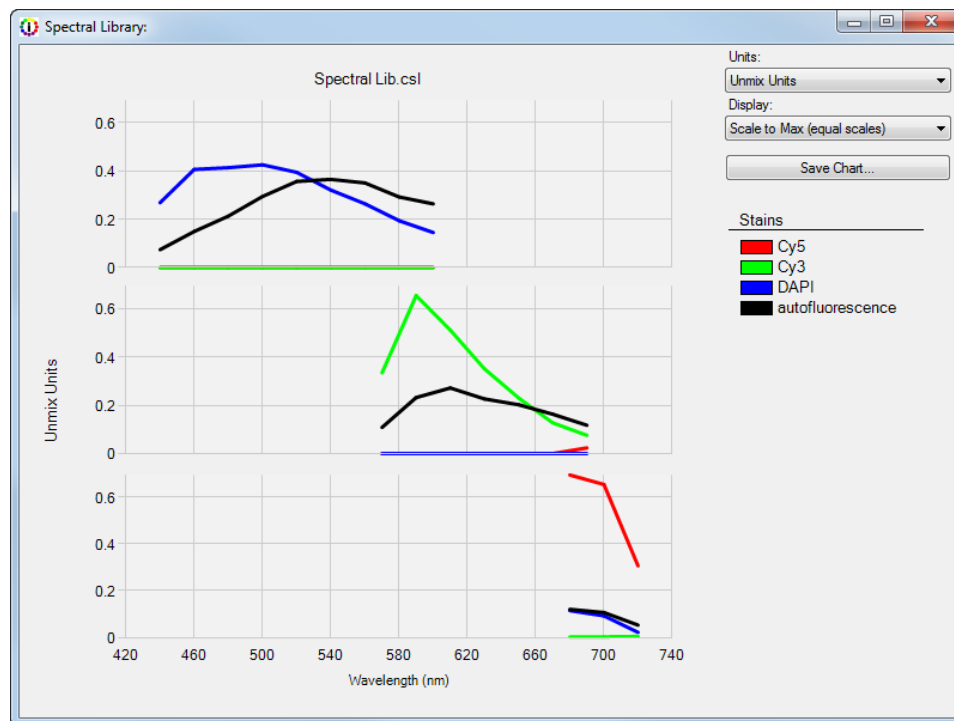


Figure 5. Spectral Library with multiple filters

Spectral Library Name: For multispectral .im3 images and RGB images, displays the name of the current spectral library above the charts. The Spectral Library name is not displayed for TRIO .im4 images because the spectral library is embedded in the image cube and does not have a separate file name.

Charts: The spectral library display can contain multiple charts; one chart for each imaging band. Each chart displays the spectral segments within an imaging band. Brightfield images only have one imaging band, so only one chart displays. After loading a library, select the check boxes for the spectra you want to use for unmixing in the Prepare Images step. The Spectral Library window automatically updates to show only the selected spectra. For TRIO (.im4) images, all spectra are selected for unmixing and the selections cannot be changed. Hovering over the line in a spectral curve displays the name, wavelength (x value), and signal strength (y value) of the nearest data point. By default, the charts open with Unmix Units and Scale to Max (equal scales) selected.

- **X Axis:** Spectral wavelength of light in nanometers.
- **Y Axis:** The units selected in the Units drop-down list.

Units drop-down list: Specifies the units on the Y Axis on the charts. Only the units valid for the spectral library are available. For example, if a spectral library does not contain Raw units, the Raw option is not available.

- **Raw Counts:** Pixel intensity in unit counts. Typical Fluorescence values are from 0 to 4095. Typical Brightfield values are from 0 to 255.
- **Unmix Units:** Displays the signal used for unmixing. Can show artifacts that are not visible in other views. Usually the best unit for evaluating a spectral library.
- **Normalized for Exposure:** Signal is displayed in counts/second. Used to compare signal strength across bands. (Fluorescence libraries only)
- **OD:** Optical Density units (Brightfield libraries only)

Display drop-down list - Specifies the Y Axis scaling option for the charts.

- **Scale to Max (equal scales):** All charts are scaled to the same value, which is the maximum signal value in all of the bands.
- **Scale to Max (varying scales):** Each chart is scaled individually to the maximum signal unit for that chart. (This option is not available when there is only one imaging band, when the library is for a brightfield image, or when the units are OD.)
- **Normalized:** Each chart is scaled individually on the Y Axis to 1. Additionally, the signals are scaled so each spectrum has a maximum of 1. (This option is not available when units are Unmix Units.)

Save Chart Button: Opens the Save Chart window to specify the location, name, and file format for saving a graphic of the charts displayed in the Spectral Library window. The chart can be saved as a .bmp, .gif, .png, .tif or .jpg and saved to a computer or network location. The graphic file includes the legend.

Legend: Displays the color for each stain in the chart. The colors are selected when the Spectral Library is created and cannot be changed in this view.

4 Common Image Analysis Tasks

Use inForm to create automated image analysis routines to accurately extract data from PerkinElmer's TRIO, Nuance, or Vectra images, or conventional RGB images (TIF, BMP, or PNG). (While it is also possible to process JPG images, this is not recommended. JPGs can contain compression artifacts that could adversely affect segmentation performance.)

Some common inForm image analysis tasks:

- inForm performs pixel-based analysis to quantitatively obtain component data. inForm can set thresholds (pixel-based) for all component signals and quantitate the thresholded component signals.
- inForm performs colocalization, which locates areas where components are simultaneously expressing on a pixel level.
- inForm identifies cells and finds nuclei, membrane, and cytoplasm, quantitates the component signal within those cell compartments and calculates consistent scoring judgments based on that quantitation.
- inForm identifies non-cellular objects, and can provide quantitative statistics.
- inForm can automatically identify tissue types based on training regions drawn by the user.

Features can be combined to perform more advanced analysis, such as computing the double positivity of cells within a cancer region, or computing the colocalization of a multi-stained tissue sample.

5 Working with Projects and Algorithms

An inForm project includes the images in the project, the algorithm to process the images, and any processing regions, training regions, or manual tissue regions.

The topics in this section describe how to create, open, and save projects, how to change the project settings, and how to open and save an algorithm.

An algorithm is used in batch mode to process images the same way each time. A project includes all the images so that you can go back and modify algorithm settings, including the trainable tissue classifier.

5.1 Creating Projects

Each time inForm starts, a new project is created automatically. The new project contains only the **Prepare Images** step and the **Export** step. To create a project with additional steps, either [Create a New Project](#)^[27] or [Configure the Open Project](#)^[28]. The name of the current project displays in the inForm title bar. Specify the desired project name when creating a new project or when configuring the project.

— Create a New Project

The steps available when creating a new project are determined by the software configuration used to create the project. See [Software Configurations](#)^[8] for information on the features available for each software configuration. (To create algorithms for use with Vectra, see [Creating Algorithms for Vectra](#)^[94]. The Vectra options are only available if inForm Tissue Finder was configured during installation for use with Vectra images.)

To create a new project:

1. Select **File > New > Project**. If an unsaved project is open already, inForm prompts you to save the current project. If you choose No, all unsaved settings are lost. The New Project window opens as shown in the figure below. Prepare Images and Export are automatically included in all projects.

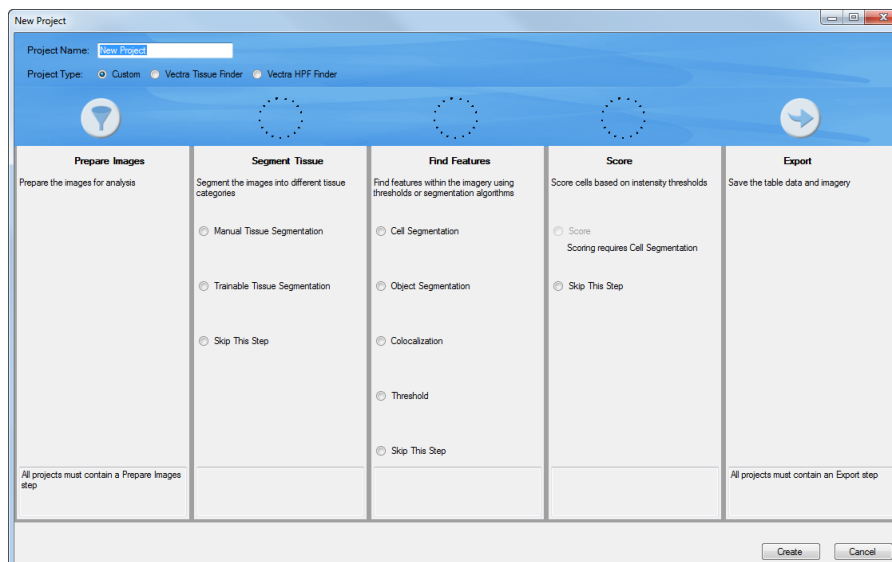


Figure 6. New Project Window

2. Type a name for the new project in the **New Project Name** text box.
3. To segment tissue, select the desired option under **Segment Tissue**:
 - a. Select **Manual Tissue Segmentation** to manually mark tissue categories on the images.
 - b. Select **Trainable Tissue Segmentation** to train the tissue segmenter to automatically identify areas as specific tissue categories.
 - c. Select **Skip This Step** to skip tissue segmentation.
4. To find specific features, select the desired option under **Find Features**:
 - a. Select **Cell Segmentation** to identify cellular features, such as finding the nucleus, cytoplasm, and membrane for each cell.
 - b. Select **Object Segmentation** to identify objects of interest.
 - c. Select **Colocalization** to identify areas on the images where selected components are colocated.
 - d. Select **Threshold** to identify the areas on the images where the strength of a component at each pixel exceeds a specific threshold.
 - e. Select **Skip This Step** to skip finding features.
5. If **Cell Segmentation** was selected, select **Scoring** to calculate score statistics for the cells.
6. Click the **Create** button to create a new project with the selected steps.
7. See [Opening Images](#)^[31] to add the desired images to the project.

— Configure a Project

You can configure a project to add or remove steps from the project. inForm retains as many settings as possible, depending on which steps are added or removed from the project. For example, if you created a project without tissue segmentation and later decide that you want the project to include manual tissue segmentation, you can modify the project to add Manual Tissue Segmentation. Settings that are not affected by the changes are retained, but some settings may need to be adjusted due to the changes to the project. You cannot reconfigure a Vectra project and you cannot convert a non-Vectra project into a Vectra project.

The steps available when configuring a project are determined by the software configuration used to configure the project. See [Software Configurations](#)^[8] for information on the features available for each software configuration.

1. Select **Tools > Configure Project** or click the **Configure** button.
2. Use the **Configure Project** window to select the desired steps. The **Configure Project** window contains the same options as the [New Project Window](#)^[22].
3. Click the **Configure** button and inForm reconfigures the project.
4. Verify that all of the desired settings are selected in each step.

5.2 Opening a Project

To open a saved inForm project:

1. Select **File > Open > Project**, select the name of the project, and click the **Open** button. The images in the project open in Gallery mode as shown in the figure below. The View Editor and the Available Images window close if either one was open in the previous project.

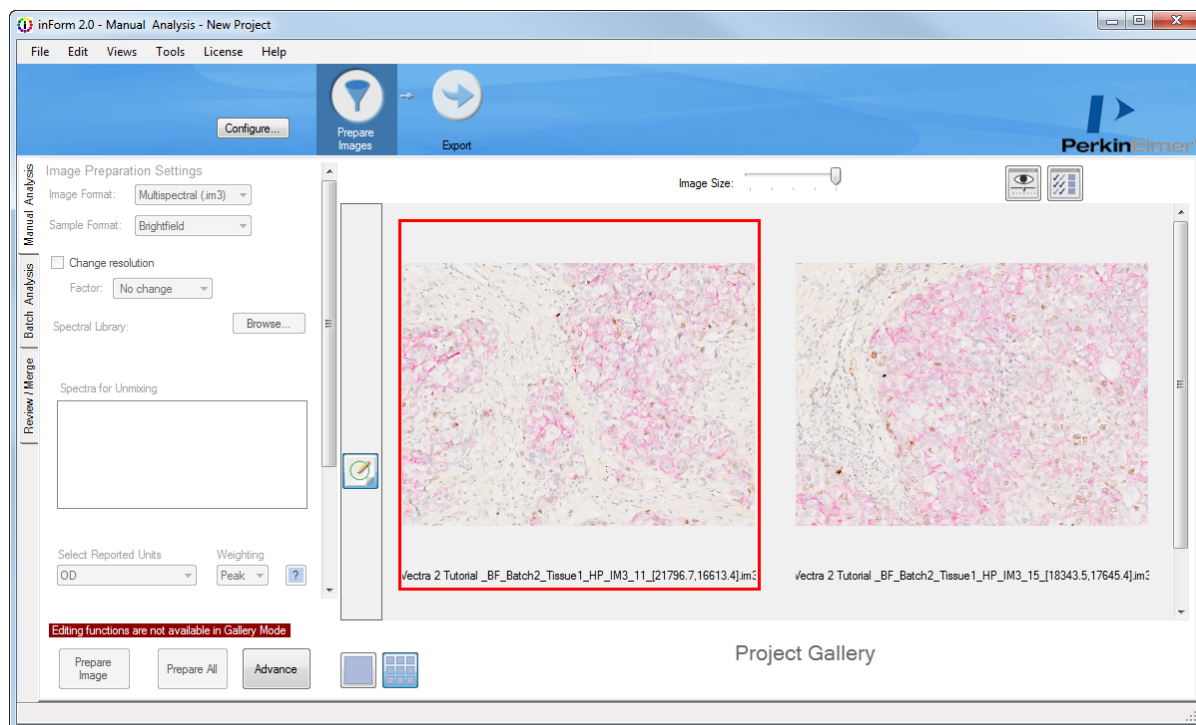


Figure 7. Gallery View

2. To edit algorithm settings, click the **Single** button to change to Single mode.

— Saving Projects

Saved projects include the images, the algorithm to process the images, and any processing regions, training regions, or manual tissue regions.

1. Select **File > Save > Project**.
2. Type a new file name, if desired, select the desired location, and click **Save**. A project file (.ifr) is saved in the specified location. A folder named <ProjectName>_TrainingImages is created in the same folder as the project file. Local copies of all the images in the project are saved in the training images folder. (To save only the algorithm and not the images, see [Saving an Algorithm](#).)

— Changing Settings

After a project has been created, the settings in the steps can be changed. Changing settings may require you to reprocess the images for later steps in the project.

5.3 Opening an Algorithm

Algorithms include all steps in processing an image: image preparation, image analysis, and export. You can load an algorithm into inForm and then open the images you want to process, or load the images first, and then apply the algorithm.

Algorithms can be used in [Batch Processing](#)^[91] to automatically process multiple images at the same time using the same analysis settings. If a project is opened for use in Batch Processing, the project's algorithm is used to process the images.

To open an algorithm, select **File > Open > Algorithm**. If a project or image is already loaded, inForm warns you that all images will be reset to their initial state. Click **Yes** to discard all training regions, tissue regions, cell regions, object regions, thresholds, and component images. Select the desired algorithm, and click **Open**.

Open the desired images if they are not already open. To prepare the image(s), click the **Prepare Image** or **Prepare All** button. To review or change the settings in each algorithm step, click the **Advance** button to advance through each step or click the step buttons at the top of the window.

When algorithm development is complete, see [Saving an Algorithm](#)^[30].

5.4 Saving an Algorithm

inForm Algorithms allow you to reuse the same settings to process compatible images or sets of images without re-selecting the settings for each new project. (Saving the project saves the images with the steps, see [Saving a Project](#)^[29].)

To save the algorithm, select **File > Save > Algorithm**. Or to save the algorithm in a protected format that cannot be edited, select **Locked Algorithm**. Type the desired algorithm name, select the desired location, and then click **Save**.

6 Opening Images

The topics in this section describe how to open images in a project and describes the viewing options available.

6.1 Opening Images in a Project

Add the images to the project as described below. The images in a project can be included in the Processing set (the images to be processed by the algorithm) and/or can be included in the Training set if training a Tissue Segmenter.

The number of images that can be opened in a project depends on the size of the images (width and height in pixels) and the operating system. Windows 7 32-bit systems can open up to 20 Nuance-sized images (1392x1040). Windows 7 64-bit systems can open up to 400 Nuance-sized images.

– Opening Images

To open the desired images:

1. Select **File > Open > Image**. The Select Image window opens. The **Files of Type** selector at the bottom of this window specifies the types of image files to display: Multispectral files (.im3), TRIO images (.im4), or other common image files such as TIFFs, BMPs, JPGs, etc.
2. Navigate to the location of the image files.
3. Select the desired image files. The image files must all be of the same image type (.im4, .im3, RGB, or Monochrome). Ctrl + click to select multiple image files. Shift + click to select a range of files.
4. Click the **Open** button to add the images to the project. The images are automatically included in the [Processing set](#)^[10].
5. To change the view of the images, see [Viewing Images](#)^[32].
6. To process only part of an image, see [Drawing Processing Regions](#)^[34].
7. To change the image resolution, convert to optical density, or unmix the images, see [Preparing Images](#)^[35].

– Opening Images Using the Available Images Window

You can add images to the project using the Available Images window.

1. Click the **Edit the list of images** button on the button bar. The [Available Images Window](#)^[21] opens.
2. Click the **Load Image** button. The Select Image window opens,
3. Select the desired images and click the **Open** button.

You can also use the Available Images window to select which images are in the training and processing sets by selecting or clearing the check boxes next to each image name.

– Adding Additional Images to a Project

After images have been added to a project, only files of the same type (Multispectral, TRIO, RGB, or Monochrome) can be added to the project. Add images to an open project either by selecting **File > Open > Image** or by clicking the **Load Image** button in the [Available Images Window](#)^[21].

– Removing Images from a Project

To remove images from the open project:

In **Single mode**, click on the thumbnail of the image to be removed to show the image in the Image Display Area. Right-click on the image in the image display area and select **Remove from Project**.

In **Gallery Mode**, right-click on an image in the gallery and choose **Remove from Project**.

Images can also be removed from the project by right-clicking on the image name in the [Available Images Window](#)^[21] and selecting **Remove Image**.

6.2 Viewing Images

Images are viewed in the Image Display area in the inForm Window. View images in Single Mode to zoom in on an image, draw regions on an image, or edit the algorithm settings. View images in Gallery Mode to compare all the images in the project or to quickly locate specific images.

Gallery Mode

To view a gallery of images, click the **Gallery** button at the lower left of the display area. Note that when in Gallery mode, you cannot edit the Step settings.



Adjust the display by moving the **Image Size** slider at the top of the Image Display Area. Move the slider to the left to fit more images in the display area. Move the slider to the right to fit fewer images in the display area.

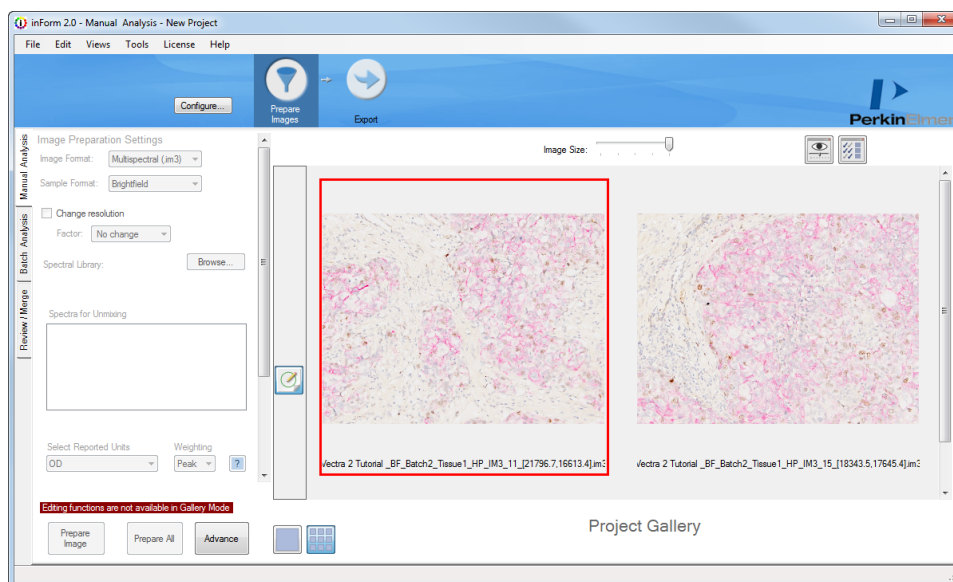


Figure 8. Gallery View

Single Mode

To view an image in Single Mode, click the **Single** button at the lower left of the display area.



Click on the thumbnail of the image to view the image in the display area.

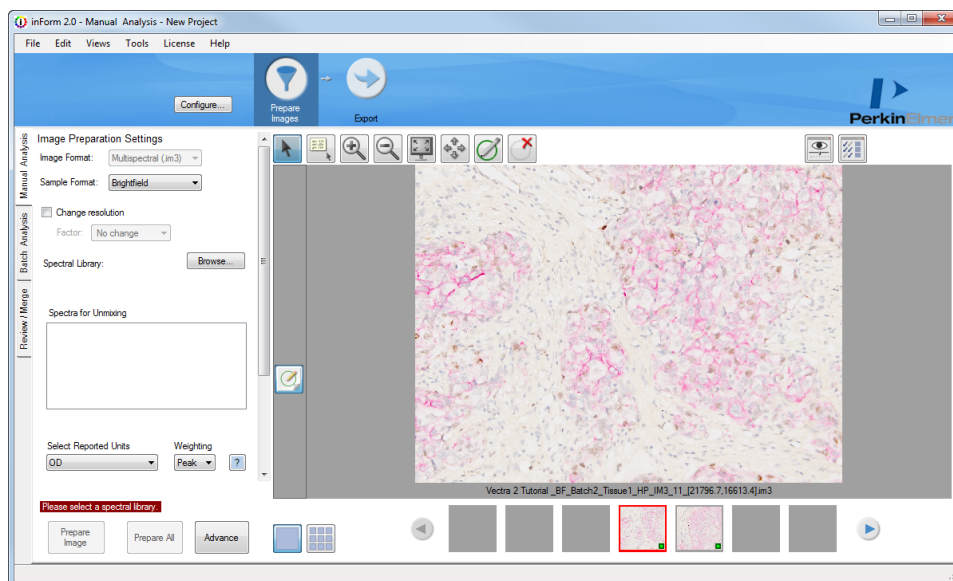


Figure 9. Single View

7 Drawing Processing Regions

Processing regions enclose areas of the image that will be processed using the steps in the project or algorithm. Areas of the image outside of the processing regions are ignored and are not included in any calculations or statistics. Processing regions are manually drawn on the images and cannot be used for [Batch Processing](#)^[9].

To draw processing regions on an image:

1. In the open project, click the **Single Mode** button, and then click on the desired image in the image thumbnails.
2. If desired, use the **Zoom In** button to zoom in on the image or the **Pan** button to move the image in the image display area.
3. Click the **Draw Processing Region** button on the toolbar.



4. Click and drag to outline the desired area of the image. Release the mouse button to connect the endpoint to the start point of the processing region. An ID number is assigned to the processing region and displays in the middle of the region. The ID number identifies the region in data tables.
5. Repeat step 4 to draw multiple processing regions on the same image.
6. Click on each image and repeat steps 2 through 5 to define all desired processing regions on each image in the project.

Deleting Processing Regions

If a processing region doesn't enclose the desired region, delete the processing region and draw a new processing region.

To delete a processing region:

1. Click the **Remove Processing Regions** button on the toolbar.



2. Click on the processing region that you want to delete. The processing region is removed and the remaining processing regions with ID numbers higher than the deleted region are re-numbered.

You can also delete a processing region by right-clicking on the processing region and selecting **Delete Region** on the shortcut menu.

To delete all processing regions on an image:

1. Right-click anywhere on the image.
2. Select **Delete Regions > Delete Processing Regions** on the shortcut menu.

8 Preparing Images

The first processing step in an algorithm is the **Prepare Images** step. This section describes how to prepare the images for segmentation or analysis by changing image resolution, converting brightfield images to optical density, and unmixing the images.

8.1 Selecting the Image Format

The **Image Preparation Settings** panel displays the Image Format, Sample Format, and Change Resolution options.

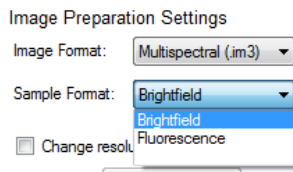


Figure 10. Image Format and Sample Format

Image Format

Before image analysis can begin, inForm needs to know the type of images in the project. Possible image types are Multispectral (.im3), TRIO (.im4), RGB, or Monochrome.

If you open a new project and then immediately add new images to the project, inForm automatically selects the correct **Image Format** (Monochrome, RGB, Multispectral (.im3), or TRIO (.im4)). If you open TIFF, BMP, or PNG images, inForm recognizes the images as either RGB or Monochrome images.

Sample Format

All images in the project must be the same sample format. For any RGB images and for Multispectral (.im3) images created with early versions of Nuance, you must select the correct **Sample Format** (Brightfield or Fluorescence). Monochrome images do not use a Sample Format. For all other image formats, inForm automatically detects the Sample Format. For some image types, you can change this selection if desired.

If desired, you can select the Image Format and Sample Format before adding images. In this case, only images that match the selected Image Format and Sample Format can be opened.

Change Resolution

Select the **Change Resolution** check box to change the image resolution. Lower the image resolution to increase the speed of training and tissue segmentation. If the set of images is so large that all of the images cannot be held in memory, lower the image resolution prior to unmixing.

8.2 Loading a Spectral Library

inForm uses a process called spectral unmixing to decompose the image into its various components. For a simple example, a color image can be unmixed into its red, green, and blue components. But more powerfully, an image can also be unmixed into its stain components such as Hematoxylin and DAB for Brightfield images, which describe the contribution of each stain to the overall signal. The contributions of each stain are based on a spectral library that shows the characteristic color distribution of light associated with each stain. A fluorescence example could consist of stain components such as DAPI and FITC; the spectral library can additionally include the color distribution associated with tissue autofluorescence.

- **TRIO multispectral .im4 images** contain the spectral library, which is automatically selected when the first .im4 file is opened in the project.
- For **Fluorescence multispectral .im3 images**, the spectral library is created in the Nuance software.
- For **Brightfield multispectral .im3 images**, the spectral library is created in the Nuance software.
- For **RGB images**, the spectral library can be created in the Nuance software or you can use the default RGB spectral library that is included with inForm.
- For **Monochrome Images**, spectral libraries are not used.

To select the spectral library:

1. For Brightfield RGB or for multispectral .im3 images acquired with early versions of Vectra, convert to Optical Density as described in [Converting to Optical Density](#)^[37] (below). Images acquired with recent versions of Vectra are already converted to Optical Density.
2. Click the **Browse** button and select the desired Spectral Library (.csl). The name of the selected library displays. The components in the library display in the **Spectra for Unmixing** list box.
3. For fluorescence images, see [Remove Haze](#)^[38] for details on removing the background haze before unmixing.
4. In the **Spectra for Unmixing** list box, select the check boxes for the components that you want to unmix.
5. To view the selected Spectral Library, click the **View** button under the Spectra Unmixing list box and see [Viewing a Spectral Library](#)^[39].

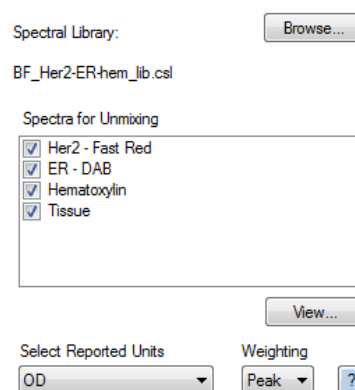


Figure 11. Load Spectral Library

— Converting to Optical Density

Brightfield multispectral and RGB images of IHC-stained samples need to be converted to optical density if the goal is to quantify stain "intensity" (optical density). Typically, brightfield multispectral images acquired with Nuance and recent versions of Vectra are already converted to optical density and do not need conversion as part of Prepare Images. Brightfield multispectral images acquired with early versions of Vectra are not converted to optical density when acquired, and require conversion as part of Prepare Images.

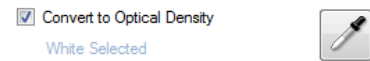


Figure 12. Convert to Optical Density

Selecting a "Reference White" for Optical Density conversion:

1. Select the **Convert to Optical Density** check box.
2. If the white areas on the image are small, zoom in on a white area.
3. Click the **White Picker** button.
4. Move the cursor (a white box) over a white region on the image and click. "White Selected" displays under the Convert to Optical Density check box. If the image appears very light, you may have clicked on an area that was not completely white. Clear the **Convert to Optical Density** check box to return to the original image, and then repeat the steps above.

Note: The optical density conversion is required if you are working with brightfield images and plan to segment cells and measure stain intensity.

— Choosing Spectra for Unmixing

The **Spectra for Unmixing** list box is populated from the selected spectral library. Select the check boxes for the signals to unmix. For fluorescence images, see the [Remove Haze](#)^[38] topic first to remove the background haze before unmixing.

For Brightfield RGB images, if you do not load a library, the spectra for unmixing default to Blue, Green, and Red.

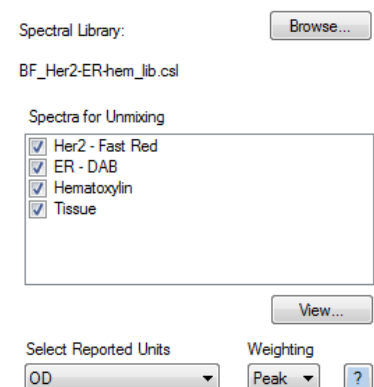


Figure 13. Spectra for Unmixing

— Removing the “Haze” Signal

In fluorescence images, image contrast and unmixing accuracy can sometimes be improved if the general haze in the image, which has a characteristic spectra, is subtracted from the images first. When creating the spectral library for the images, create the background or “haze” spectrum and add it to the library. When the library is loaded, you can select the haze spectrum and remove it from the images.

To remove haze:

1. Check the **Remove Haze** check box.
2. Select the **Haze Spectrum** to remove.

Haze is removed numerically (without scaling or other changes in value), wavelength-by-wavelength, from every pixel of the image. This alters the spectral shape of the remaining signals and their overall intensity. The magnitude of these changes depends on the intensity of the spectral signal being subtracted. Typically, when background spectra are small compared to the signals, spectral library entries derived from unsubtracted data sets can still be used, but this should be validated when quantitative results are required. Ideally, the remaining spectra in the library should also have the haze spectrum subtracted (in Nuance).

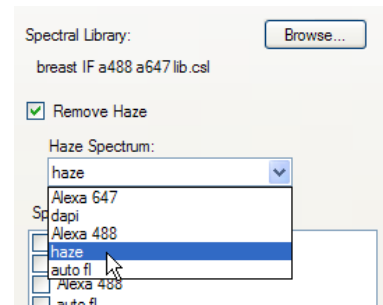


Figure 14. Remove Haze Spectrum

8.3 Viewing a Spectral Library

The Spectral Library window displays a chart for each spectral segment in a spectral library.

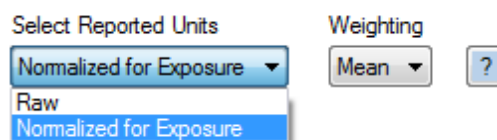
To view the spectral library:

1. Open the desired images and spectral library.
2. Select the spectra that will be used to unmix the images in the **Spectra for Unmixing** list box.
3. Click the **View** button below the Spectra for Unmixing list box to open the [Spectral Library Window](#)^[24].
4. Select the desired **Units** to display the stains in the charts. See [Spectral Library Window](#)^[24] for complete descriptions.
5. Select the desired **Display** options for the chart(s).
6. To save an image of the chart with the currently selected display options, click the **Save Chart** button, type the desired name for the image file, select the desired image format, and click the **Save** button.
7. Click the Close (X) button in the upper right corner to close the Spectral Library window.

If a chart cannot be displayed because no spectra are selected or there are no compatible units, a message displays in place of the spectral charts.

8.4 Component Units

The components produced by unmixing are calculated using the settings selected in the **Select Reported Units** and **Weighting** drop-down lists. The Reported Units can be: Raw counts, counts Normalized for Exposure, or OD (optical density) units. The Weighting can be Total, Mean, or Peak.



Brightfield versus Fluorescence

Brightfield and Fluorescence images use different measuring schemes because of the inherent differences of the images.

- For Brightfield images, darker pixels indicate a higher expression of the biomarker.
- For Fluorescence images, brighter pixels indicate a higher expression of a biomarker or reporter protein.

Therefore we use optical density for brightfield images and fluorescence counts (either raw or normalized) for fluorescence images.

Select Reported Units

Monochrome (black and white) images and **RGB** color images produce signals in counts.

For **multispectral images that are not converted to optical density**, component signal counts are either *raw* or *normalized for exposure*.

- **Raw Counts** reports the measured signal associated with a given component (such as DAPI, FITC, etc.).
- **Normalized for Exposure** reports the number of counts in the image, after dividing by a factor that accounts for all the acquisition settings in use (gain, binning, bit-depth, and exposure time). Using Counts Normalized for Exposure allows you to compare images taken using different camera settings or exposures, with the assurance that signal levels indicate the actual brightness at the sample, regardless of the instrument settings.

Counts Normalized for Exposure = counts / {2^{bit depth} x exposure time x gain x binning area}, where bit depth is the bit depth of the imagery, typically 8-bit (256 levels of gray) or 12-bit, exposure time is in seconds, gain is the gain setting of the camera, and binning area is 1 for 1x1, 4 for 2x2, etc.

The values are small when using Mean weighting, and it may be hard to develop an intuition about what is normal. Selecting Total weighting (default) produces larger values.

For **brightfield images that are converted to Optical Density**, inForm produces unmixed component signals in **OD (Optical Density) units**. Optical Density indicates the amount of absorbing material present at a location. Optical Density = $-\log_{10}(\text{pixel value} / \text{white reference pixel value})$.

Weighting

Specifies whether to use the Mean, Peak, or Total signal to calculate the displayed units.

Total: The sum of all signals of the selected component across all wavelengths. Available for all images except images converted to Optical Density. (Default for fluorescence images.)

Mean: The average of all signals of the selected component across all wavelengths. Available for all units except Counts for Monochrome images. This is the equivalent of the settings in versions of inForm prior to V2.0.2. Examples: For RGB color images, inForm reports the average signal across the three color planes (red, green, and blue). A pure blue pixel with 240 counts would have a component strength of 80 (= 240 counts / 3 planes). For multispectral images, the signal is averaged across all wavelengths measured. So a pixel having a DAB signal of OD = 0.60 in the blue, OD = 0.20 in the green, and OD of 0.10 in the red would report a signal of 0.30 OD units (= [0.60 + 0.20 + 0.10] summed OD / 3 planes).

Peak: The peak signal of the selected component across all wavelengths. Available for images converted to Optical Density and for fluorescent multispectral images. (Default for brightfield images.)

Weighting Example

The simplified example below explains the difference between the three weighting options. (The example uses only two fluors and ignores noise and other sources of error.)

This example uses a spectral library with just two elements, Fluor A and Fluor B, and eight wavelengths (420, 460, 500, 540, 580, 620, 660, 700). The spectral library is shown in the figure below.

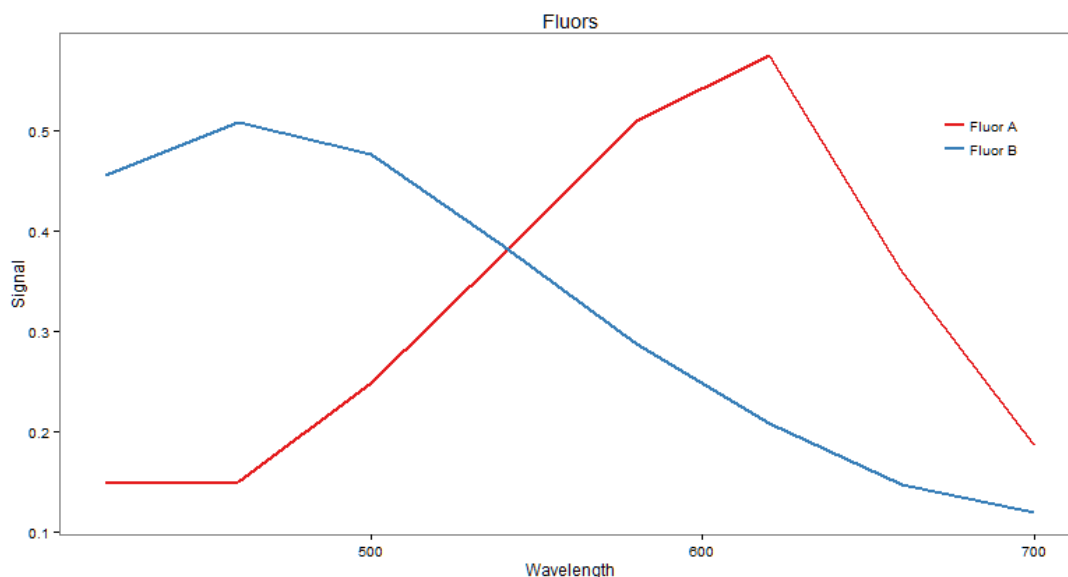


Figure 15. Spectral Library Example

If an image pixel expresses one part Fluor A and three parts Fluor B, the signal at the pixel is shown in the figure below.

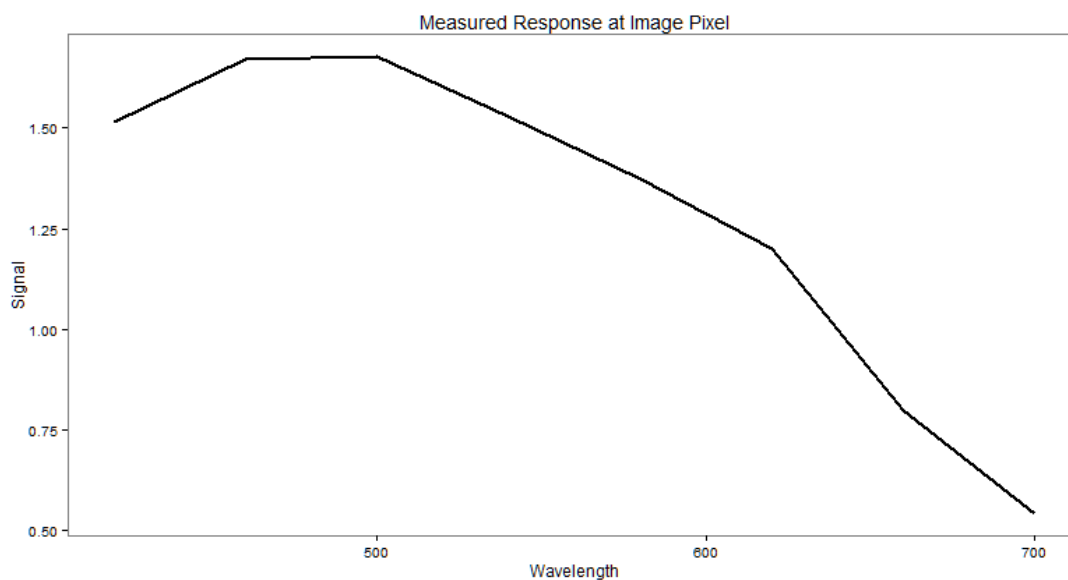


Figure 16. Signal at a Pixel

Unmixing recovers the original spectra, scaled according to the measured expression at the pixel as

shown in the figure below.

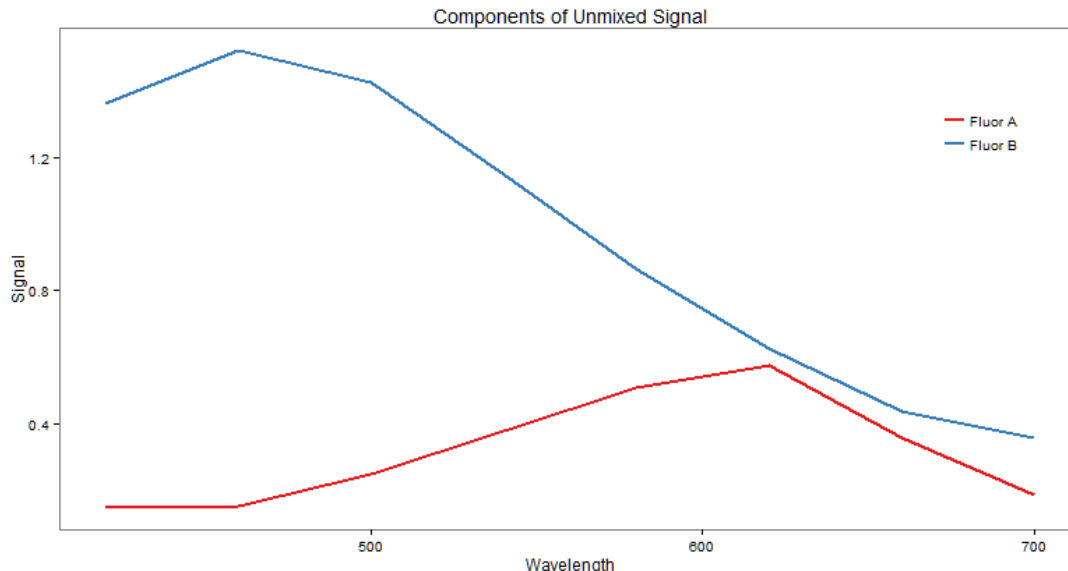


Figure 17. Original Spectra, Scaled

The challenge for the unmixed component image is to represent the unmixed spectrum using a single value. The three weighting options correspond to three possibilities for how to do this.

- Peak weighting represents the spectrum as the signal level at the brightest wavelength of the spectrum. In the example, this gives component values of

Fluor A = 0.5739

Fluor B = 1.5216

- Total weighting represents the spectrum as the sum of the signals at all wavelengths. In the example, this gives component values of

Fluor A = 2.551

Fluor B = 7.7514

- Mean weighting represents the spectrum as the average signal across wavelengths. In the example, this gives component values of

Fluor A = 0.3189

Fluor B = 0.9689

Notice that Total weighting is the Mean weighting multiplied by the number of wavelengths (eight in this example).

The figure below shows scaled spectra annotated with the weighting values:

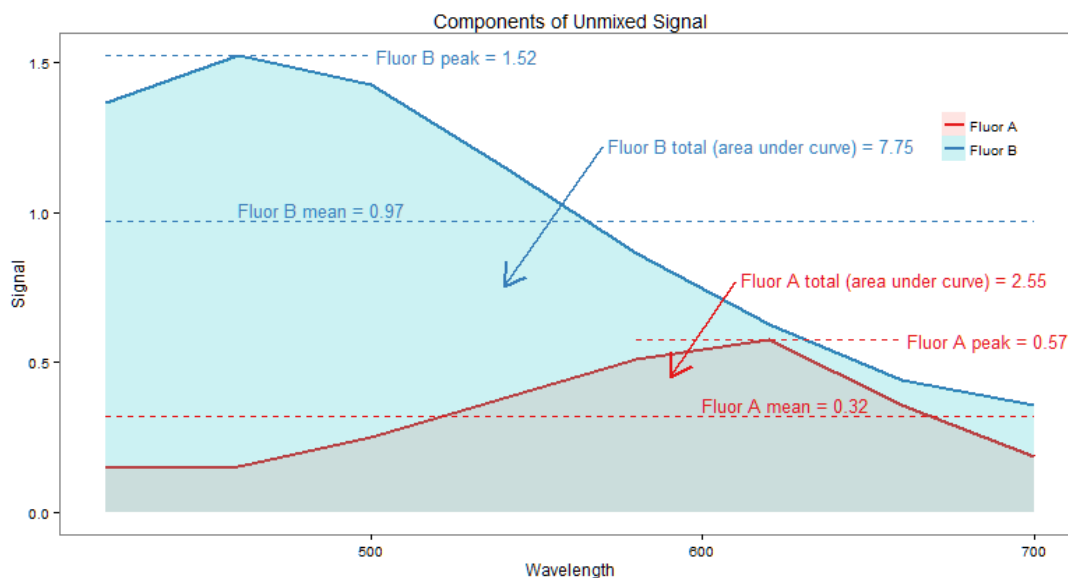


Figure 18. Weighting Values

8.5 Completing the Prepare Images Step

After all of the desired settings have been selected in the Image Preparation Settings panel, process the images using any of the methods below and then advance to the next step in the project.

- Click the **Prepare Image** button to apply the selected settings to the currently selected image.
- Click the **Prepare All** button to apply the selected settings to all images open in the project.
- Click the **Advance** button to apply the selected settings to all images open in the project and then display the next step in the project.
- Click the next step in the project in the [Step Bar](#) ¹⁴ at the top of the inForm window. All images are processed using the current settings, and then the settings panel for the next step displays.

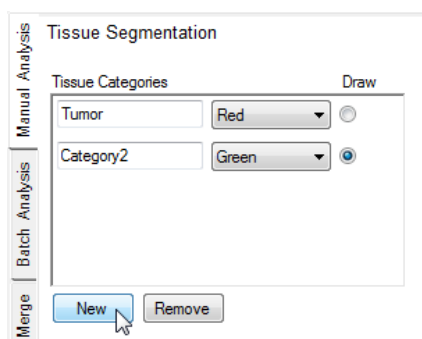
9 Manual Tissue Segmentation

The topics in this section describe how to manually draw tissue categories on an image. The tissue categories can then be used to generate data or can be used during the Cell Segmentation, Scoring, or Object Counting steps. Algorithms that include a Manual Classification step cannot be used in Batch processing.

9.1 Creating Tissue Categories

Tissue categories divide the image into specific areas that correspond to specific types of tissue (for example, cancer, necrosis, stroma, etc.) or occasionally other structures of interest. The Manual Classification step (available in inForm Basic Analysis or higher) enables you to draw the appropriate tissue categories over areas of each tissue type on the images. Cell segmentation, scoring, and object counting can all be restricted to a specific tissue category.

1. Click the **Manual Classification** step to display the *Tissue Segmentation* panel.
2. Click the **New** button under the Tissue Categories section to create a new tissue category.



3. To change the tissue category name, highlight the name and then type the desired name for the tissue category.
4. To change the display color for the tissue category, click the **Color** pull-down and select the desired color.
5. Repeat steps 2 through 4 to create all of the desired tissue categories.
6. See [Drawing Tissue Category Regions](#) ⁴⁴.

9.2 Drawing Tissue Category Regions

Draw tissue categories around the areas on the image that contain each defined tissue type. A single area on the image cannot belong to more than one tissue category.

To draw tissue category regions on an image:

1. Click the **Draw** option button next to the desired tissue category.
2. Click and drag to outline the desired area on the image.
3. If the region intersects another region in the same tissue category, the two regions are merged into one region.

4. If the new region intersects a region in a different tissue category, the area becomes part of the new tissue category.
5. The unselected parts of the image file are covered with a gray mask. This may make it difficult to see the image to select additional tissue categories. To temporarily hide the tissue categories and the gray mask, click the **Show/Hide Tissue Segmentation Map** button.



After the next tissue category is drawn on the image, the tissue segmentation map displays again.

6. See [Editing Tissue Category Regions](#)^[45] if necessary.
7. When all of the desired tissue categories have been drawn, continue with the next processing step in the project. Either click the **Advance** button or click the next step button.

9.3 Editing Tissue Category Regions

After tissue categories have been drawn on an image, you can edit the regions to add areas to a region, remove areas from a region, or remove individual pixels from a region.

To add an area to manually selected tissue categories:

1. Zoom in or out on the image to the desired magnification.
2. Click the option button next to the tissue category that you want to add an area to.
3. If desired, use the **Tissue Segmentation Map** button to show or hide the tissue masks. Hiding the tissue masks makes it easier to see the colors on the original image.



4. If Processing Regions have been defined, use the **Process Regions** button to show or hide the process region masks.



5. Click and drag to outline the area to be included, intersecting the defined tissue classification areas as desired.
6. Release the mouse button to connect the end point to the start point. The outlined area is assigned to the specified tissue category.

To remove an area from the tissue category:

1. Click the **Select Regions to Unclassify** button.



2. Click and drag to enclose the area from which you want to remove the tissue classification.
3. Release the mouse button to connect the end point to the start point. The outlined area is removed from the tissue category.

To remove single pixels from the tissue category:

1. Zoom in on the image.
2. Click the **Select Pixels to Unclassify** button.



3. Click on a single pixel to remove it from the tissue classification region.
4. Click and drag to remove a line of pixels from the tissue classification region.

10 Trainable Tissue Segmentation

The topics in this section describe how to automatically classify tissue using the tissue segmenter. To set up the tissue segmenter, add tissue categories and draw training regions on images in the training set, train the tissue segmenter, segment images, and then fine tune the segmenter based on initial results.

10.1 Adding Tissue Categories

Tissue categories divide the image into specific areas that correspond to specific types of tissue (for example, cancer, necrosis, stroma, etc.) or occasionally other structures of interest. The Segment Tissue step (only available in inForm Tissue Finder) enables you to draw training regions around representative areas of tissue on images and then train the tissue segmenter to automatically locate similar regions of tissue in each image.

The **Tissue Segmentation Training** panel is used to define tissue categories, train segmenters, and segment images in the image processing set.

— Creating Categories

1. Click the **Segment Tissue** step to display the *Tissue Segmentation Training* panel.
2. Decide how many tissue categories you want to segment the images into.
3. Click the **New** button and type the desired name of the tissue category in the text box.
4. Create the desired number of tissue categories for the images in the project.
5. To change the display color for a tissue category, click the **Color** pull-down and select the desired color.

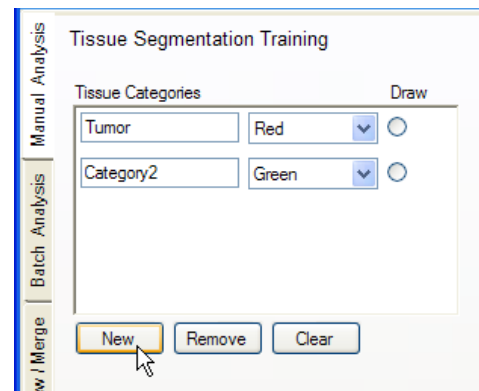


Figure 19. Creating Tissue Categories

— Deleting Categories

To remove a category, click the category's radio button and click the **Remove** button.

To clear all tissue categories and start over, click the **Clear** button.

10.2 Drawing Training Regions

Training regions teach the tissue segmenter which cells should be included in each tissue category. It is important that you already know what to look for and are able to visually recognize structures characteristic of the tissue category. For example, if there are tumor cells present in any image that are negative (i.e., not stained), be sure to include those also. It is important to train on the full range of appearances of the tissue category, as in the example, where negatively-stained as well as the positively-stained cells are included.

To draw the training regions:

1. In the Tissue Segmentation Training panel, click the Draw radio button for a tissue category.
2. Click and drag to draw regions around groups of cells. (For example, if the tissue category is named *Tumor*, draw regions around groups of tumor cells).

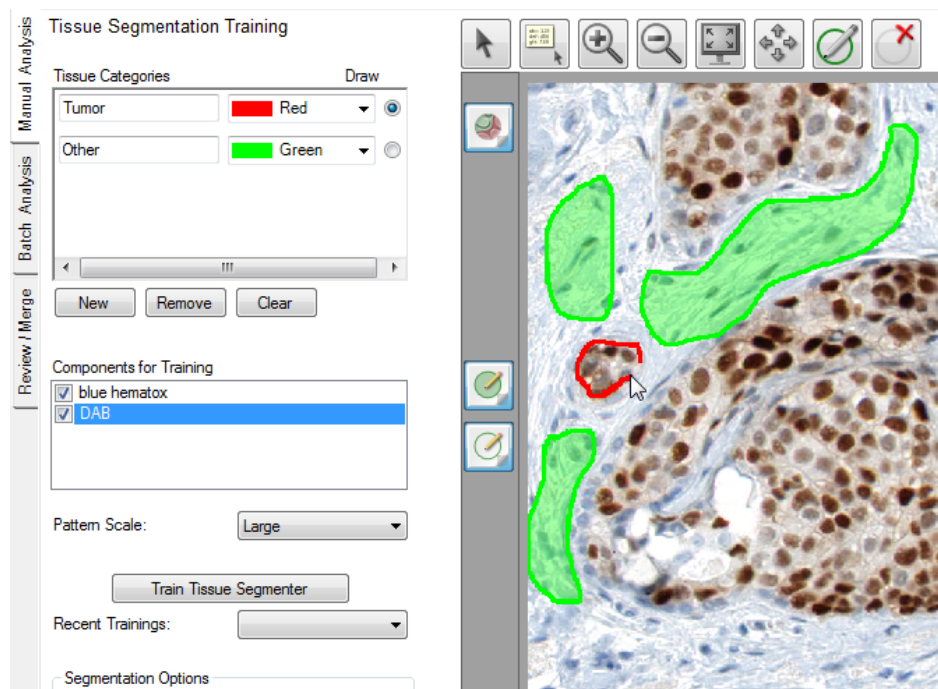


Figure 20. Drawing Training Regions

3. To delete a region, either click the **Delete Region** button and click inside the region to be deleted, or right-click on the region and select **Delete Region** from the pop-up menu. (This menu also lets you delete all training regions or all within a selected category.)
4. When you are finished drawing regions for the first category, switch to the next category (e.g., non-tumor regions) and draw regions around cells characteristic of the next tissue category. Be sure to capture visual anomalies such as areas slightly out of focus, folds, bubbles, etc. Also capture stroma regions that have differing qualities of shape or orientation.
5. Repeat this process for all of the images in the training set.
6. When all training regions have been drawn, see [Training the Tissue Segmenter](#)⁴⁹.

10.3 Training the Tissue Segmenter

Only images that are included in the Training set are used to train the tissue segmenter. Images that have been removed from the training set are not used to train the tissue segmenter, even if the images include training regions.

— Selecting Components for Training

Select the components that you want to use for training the tissue segmenter.

Components that provide distinguishing pattern information that is independent of signals to be quantified are preferred. For example, the counterstain component plane (e.g., DAPI or Hematoxylin) should be tried first for training because the counterstain reveals the structure of the tissue, and is less likely to produce a segmenter biased by the presence of the molecular marker you are trying to quantify. For example, if measuring the positivity of cells stained with Alexa Fluor® in a sample stained with both DAB and Alexa Fluor, you would not want the segmenter to be dependent on the Alexa stain because that would bias the outcome.

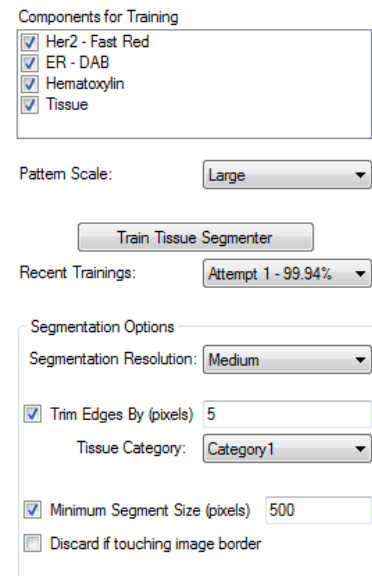


Figure 21. Components for Training

On the other hand, sometimes in brightfield applications where the presence of an IHC stain such as DAB blocks the staining of the counterstain, using both the counterstain and the IHC stain component planes for training yields a more robust tissue segmenter, even for the negative cases. In these cases where the target label is included in the training, it is important to include abundant negative cases in the training set.

Also, you should generally avoid using components that provide information unrelated to the desired segmentation.

— Selecting a Pattern Scale

The recommended approach is to start with the default *Large* pattern scale. Once you have drawn training regions and trained a tissue segmenter, if segmentation accuracy for the training regions is low (<85%), increasing **Pattern Scale** may improve accuracy. However, the pattern scale needs to be compatible with the size of the training regions. Pattern scale refers to the spatial extent of patterns that are measured to support machine learning and image analysis.

If the selected pattern scale (e.g., *Maximum*) is too large for the drawn training regions, a grey mask displays on training regions that are too small after training and those training regions are not used to train the tissue segmenter.

You should use as large a pattern scale as is compatible with the training regions that you draw. If most of the training regions have gray masks over them after training, reduce the pattern scale (.e.g., from *Large* to *Medium*), and then retrain.

For example, if analyzing tumors in tissue sections in a 20x magnification image, the architecture is fundamentally on a large scale, and you probably want to select *Large*. For items with inherently fine scale structures, select *Small* or *Medium*. An understanding of the different scales can be learned by seeing what size training regions are masked with grey after training.

— Train the Segmenter

Click the **Train Tissue Segmenter** button to begin training the segmenter. The training process may take some time, so be patient.

The percent accuracy of the new tissue segmenter displays. If, for example, the segmenter was 99% accurate, this would mean that if the new segmenter was to be applied to all of the images used in training the segmenter, it would classify the pixels in the training regions with 99% accuracy. (Fewer than 1% of the pixels in all the drawn regions would be misclassified.)

The software continues to try to improve the accuracy. Click the **Stop** button to stop training when an acceptable level of accuracy is reached or the accuracy has stabilized. In general, segmenters should be at least 80% accurate. If the accuracy reaches 100%, a **Done** button displays to close the Algorithm Training dialog.

— Recent Trainings

The **Recent Trainings** box records a history of segmenters trained using the current segmentation settings. For example, the first time you train is named "Attempt 1," the second training is named "Attempt 2," and so on. To re-classify the images using a previous segmenter, select the desired training attempt (i.e., segmenter) from this drop down box. If you change any settings, the history of segmenters is cleared.

— Segmentation Resolution

The **Segmentation Resolution** determines the resolution of the tissue category segmentation. There is a trade-off between resolution and segmentation speed, with an approximately 4x increase in segmentation speed for every step in resolution reduction. Select *Coarse* if you want to segment the images more quickly. Although the edges of the segmented regions appear pixelated, the segmenter still accurately differentiates structures well (e.g., tumor from stroma). Select *Fine* if the segmented regions need to have smoother edges, such as images for use in publications. Segmenting with *Fine* resolution takes longer than with *Coarse* resolution. Also, if you selected a small Pattern Scale, the Resolution must be set to *Medium* or finer.

– Segmenting the Tissue in the Training Images

Once you have trained the segmenter, you can segment the tissue in the current image or all of the images in the training set. You can segment just the current image first, to test the tissue segmenter. Then make any desired adjustments to the training parameters and retrain the segmenter. To see the Tissue Category mask on all images, click the **Segment All** button. When you are ready, advance to the next step in the project.

– Fine Tuning the Segmenter

Observe the training regions following segmenter training. As mentioned above, if the software grays out any region, this indicates that the region is too small for the selected **Pattern Scale** and was not used to train the tissue segmenter. You can ignore this if segmentation performance is adequate, delete the region and redraw a larger one, or try selecting a smaller pattern scale.

The **Trim Edges** option can be used to "erode" a tissue segmentation by removing the specified number of pixels from the periphery of the selected tissue category, making it smaller. This is useful if the tissue segmenter has a tendency to include pixels extending beyond the edges of objects or tissue categories of interest (e.g., tumor or vessels). For example, if you notice cancer regions extending into non-cancerous areas, select the **Trim Edges** option, choose the cancer tissue category, and enter the number of pixels you want to trim from the borders of the cancer regions.

The **Minimum Segment Size** option is an adjustable parameter (in pixels) that removes segments that have fewer pixels than this value. The removed segment is replaced by the tissue segment that is most abundant around the segment that was removed.

If the **Discard if Touching Image Border** check box is selected, tissue regions touching the edge of the image or process region are discarded. If not selected, all tissue regions are included.

10.4 Adding Images to the Training Set

When selecting images for the training set, it is recommended that you start with one image per distinct appearance. The training set should represent the range of staining levels and tissue architectures that you want to train the tissue segmenter to identify.

Whenever you draw a training region on an image (see [Drawing Training Regions](#)^[48]), inForm automatically adds the image to the training set. You can also add or remove images from the training set manually as described below.

1. Click the **Image List Editor** button to open the [Available Images Window](#)^[21].



2. Select the **Training Set** check box next to the images to add the image to the Training set.
3. Clear the **Training Set** check box next to an image to remove the image from the Training set.
4. Close the Available Images window.
5. When you switch to Single mode, a small blue square on each thumbnail image at the bottom of the screen (see figure) indicates images are included in the training set. A green square

indicates that the image is in the processing set.

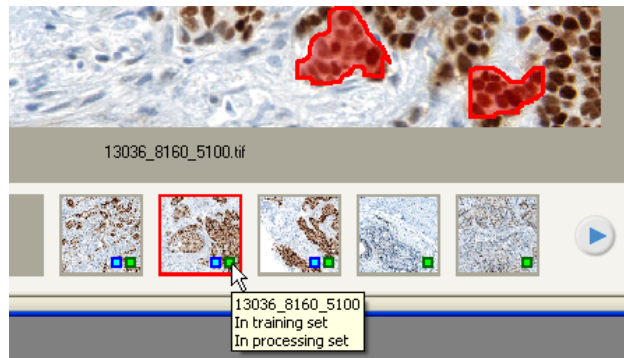


Figure 22. Training Set and Processing Set Indicators

6. See [Drawing Training Regions](#) ^[48] to add training regions to the desired images in the training set.

10.5 Editing Masks and Re-Training

After reviewing the segmented images, you might discover that some structures were not segmented correctly, or the segmenter may have selected some unwanted regions. The Tissue Segmentation masks should accurately cover their respective tissue categories.

To correct the tissue masks, you can either edit the mask manually, or you can re-train the tissue segmenter to detect the desired regions.

Manually Editing the Tissue Segmentation Mask

If the experiment or study involves few enough images, you can manually edit the Tissue Segmentation mask. Manual editing does not change the tissue segmenter and if the images are re-segmented, the changes to the Tissue Segmentation masks are lost.

To manually add areas to a Tissue Segmentation mask:

1. Click the **Edit Tissue Segmentation** button on the toolbar.
2. From the drop-down list, select the tissue segmentation mask that you want to edit.
3. Draw around the areas on the image that you want to include in the selected Tissue Segmentation mask.
4. To continue processing the images using the edited Tissue Segmentation masks, advance to the next step without re-segmenting the images.
5. If the project contains a cell segmentation step and cell segmentation was performed before the mask was edited (see [Segmenting Cells](#) ^[59]), you should click the **Segment Images** button in the **Cell Segmentation** editor to re-segment the cells in the edited tissue segmentations.

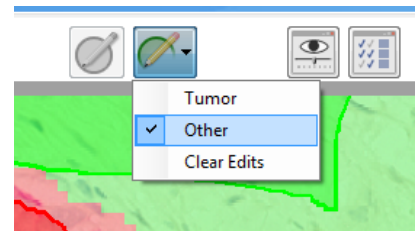


Figure 23. Editing Tissue Segmentation Masks

Re-Training the Tissue Segmenter

If some areas of the image were not classified as the desired tissue type, you can re-draw the training regions around the areas that were incorrectly classified and re-train the Tissue Segmenter.

To re-train the tissue segmenter:

1. In the Tissue Segmentation Training panel, click the Draw radio button for the tissue category that you want to draw additional training regions for.
2. Draw training regions around areas that were incorrectly classified.
3. Re-train the tissue segmenter and then re-segment the image. Verify that the desired regions are now correctly classified in the desired Tissue Segmentation mask.
4. To see the tissue segmentation on all images, segment all images. (Note that re-segmenting an image after retraining the tissue segmenter deletes any manual edits to the Tissue Segmentation mask.)

10.6 Completing the Automated Tissue Segmentation Step

After all of the desired settings have been selected in the Tissue Segmentation Training panel and the Tissue Segmenter has been trained, process the images using any of the methods below and then advance to the next step in the project.

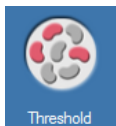
- Click the **Segment Image** button to segment the currently selected image.
- Click the **Segment All** button to segment all images open in the project.
- Click the **Advance** button to display the next step in the project. (The tissue is segmented the next time images are processed.)
- Click the next step in the project in the [Step Bar](#)¹⁴ at the top of the inForm window. The settings panel for the next step displays. (The tissue is segmented the next time images are processed.)

11 Threshold

Use the Threshold step to set threshold values for any of the unmixed components in an image and then to view the regions on the image that are above the threshold. Statistics are calculated for the thresholded regions and are displayed in the Quant Data table.

To set the thresholds and calculate the threshold statistics for the images:

1. Click the **Threshold** button in the step bar.

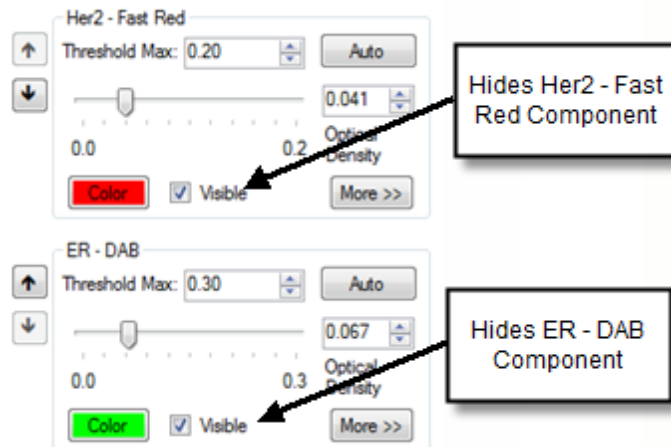


2. In the **Components to Threshold** list, select each of the image components you want to create a threshold map for.
3. Click the **Auto** button to calculate a preliminary value for the Threshold Max and to choose a preliminary Threshold value. The **Threshold Max** is set to the value of the brightest pixel in the component. The slider range is set to (0 - Threshold Max). The threshold is set to an optimal value, based on the component values. (The threshold max and threshold can be set manually without clicking the Auto button, if desired.)
4. Set the desired Threshold for each unmixed component. Move the slider, type the desired value or click the up and down arrow buttons to select the threshold. The image display shows the areas that are above the threshold value as the slider is adjusted.
5. To change the color displayed for the regions, click the **Color** button and select the desired color.
6. To change the minimum size of the thresholded regions:
 - Click the **More** button under the name of the Threshold map that you want to change.
 - Set the desired value for **Minimum Connected Pixels** by either clicking the Up or Down arrow buttons or typing the value in the text box. The Minimum Connected Pixels specifies a minimum region size based on the number of pixels. Only regions that are larger than the Minimum Connected Pixels value are considered. Regions with fewer than this number of pixels are ignored.
7. If there are multiple images in the project, either select each image and apply the current settings to the image by clicking the **Accept for Image** button or click the **Accept for All** button to apply the Threshold settings to all of the images in the project. Verify that the threshold settings are appropriate for all images in the project and adjust if necessary.
8. If desired, [change the threshold map view](#)^[55] or [edit the threshold maps](#)^[56].
9. To view the statistics for the generated regions in the Quant data table, see [Viewing the Quant Data Table](#)^[86], or to export the data, see [Exporting the Data](#)^[89].

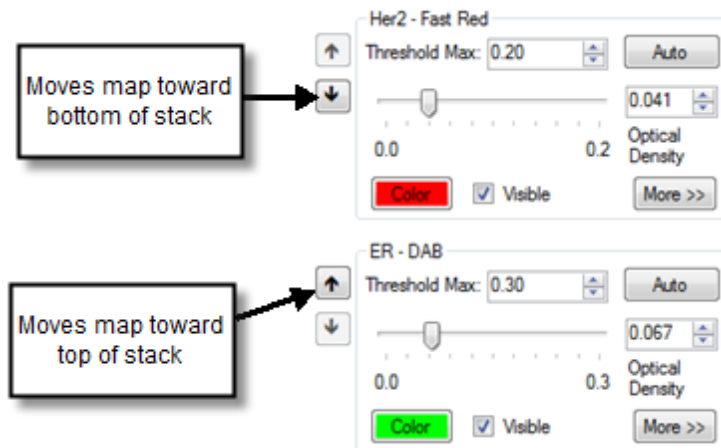
Changing the Threshold Map View

The threshold maps for each component are stacked in layers on top of the original graphic, with the top component in the Simple Threshold Settings panel as the uppermost layer in the image display. You can change the order of the threshold maps or hide threshold maps.

1. To hide a threshold map, clear the **Visible** check box under the component that you want to hide.



2. To move a threshold map up or down in the stack of maps, click the **Up** or **Down** arrows on the left of the Color button.



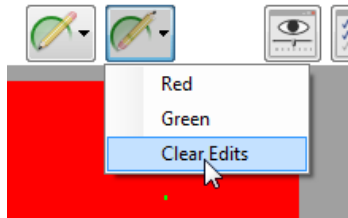
Editing Threshold Maps

Threshold maps can be edited manually. Additional areas can be drawn onto the threshold map or undesired areas can be erased from the threshold map. This procedure describes how to use the editing tools on the Simple Threshold Settings panel. The Add Thresholded Regions and Erase Thresholded Regions buttons on the toolbar can also be used.

1. To view editing options, click the **More** button under the name of the Threshold map that you want to edit.
2. To draw additional threshold regions, click the **Add** button under the component name and draw the desired regions on the threshold map.
3. To remove threshold regions, click the **Erase** button and draw around the area to be removed. Only the threshold areas for the selected component are erased.
4. To clear all manual edits from a threshold mask, click the **Clear** button under the name of the component. Only the manual edits on the threshold map for that component are cleared.

To clear all manual edits from all threshold maps:

1. Click the down arrow next to the **Erase Threshold Regions** button and click **Clear Edits**.

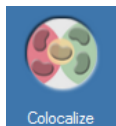


2. Click **Yes** in the Clear Mask Edits window.

12 Colocalization

Colocalization is a pixel-based analysis used to find overlapping components in images based on thresholds set for each component. Colocalization of multiple markers can be determined and displayed both visually and statistically. It is designed for analyzing and quantitating molecular markers and can be used to determine the amounts of colocalization of multiple markers.

1. Click the **Colocalization** button in the step bar.



2. Under **Markers for Colocalization**, select the markers for which you want to see overlap (colocalization) and positivity percentages.
3. Under **Denominator (Counterstain)**, select the marker(s) to use as the counterstains. To see colocalization of markers throughout the entire image and not just within a particular counterstain, select All Image Pixels.
4. For each component selected in the Markers or Denominator, a **Threshold** settings area displays below the Denominator check boxes.
5. To adjust the Threshold for each component:
 - a. Click the **Auto** button to calculate a preliminary value for the Threshold Max and to choose a preliminary Threshold value. The **Threshold Max** is set to the value of the brightest pixel in the component. The slider range is set to (0 - Threshold Max). The threshold is set to an optimal value, based on the component values. (The threshold max and threshold can be set manually without clicking the Auto button, if desired.)
 - b. Set the desired Threshold for each unmixed component. Move the slider, type the desired value, or click the up and down arrow buttons to set the threshold. The image display shows the areas that are above the threshold value as the slider is adjusted.
 - c. To change the color of a threshold mask, click the **Color** button and select the desired color.
6. To view the threshold for each component, clear the **Visible** check box for all components

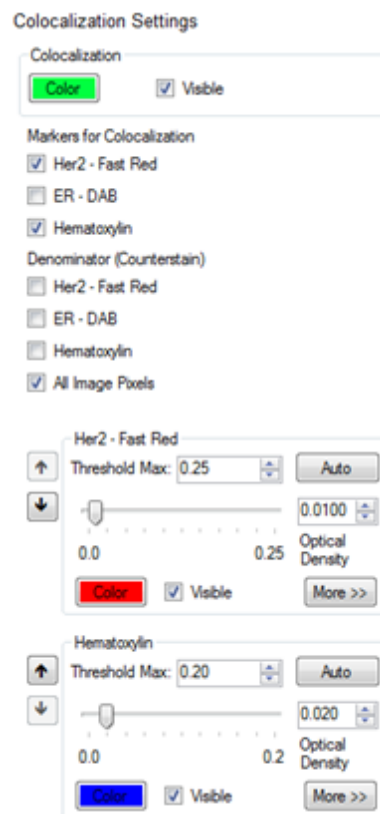


Figure 24. Colocalization Settings

except the component that you are adjusting. Clear the **Visible** check box for Colocalization. Adjust the Threshold for the component until all of the desired areas are marked as positive.

7. If there are small regions that are marked as positive that should not be included, or if smaller regions should be marked as positive and are not, click the **More** button and adjust the **Minimum Connected Pixels** value as desired.
8. Under **Colocalization**, click the **Visible** check box to show the colocalization map. If desired, click the **Color** button and select the color to use to indicate areas of colocalization.
9. To view the Colocalization statistics, see [Viewing the Quant Data Table](#)^[86] and [Viewing the Colocalization Data Table](#)^[84].
10. To export the data, see [Exporting the Data](#)^[89].

13 Segmenting Cells

Cell segmentation locates individual cellular or subcellular objects within an image field or within a selected tissue category if tissue has been segmented. By first identifying the location of all cell nuclei, inForm is able to identify cells and their associated cytoplasm and membrane. Once individual cells are located, the set of pixels associated with each cell is identified so that marker signals can be extracted for analysis and reporting.

Note: Cells are considered to be an assembly of associated cellular components (nucleus, cytoplasm, and membrane). If you are segmenting tissue, some cells may straddle two segmented tissue regions. If a cell is not entirely contained within a single region, the “containing region” is undefined, and the Region ID in the Data tables is #N/A (see [Displaying the Extracted Data](#)^[73]).

The Segment Cells step is only available in inForm Cell Analysis and inForm Tissue Finder.

Use the **Segment Cells** panel to set parameters for segmenting cells into their respective categories so that data can be extracted and analyzed on a per-cell basis.

To segment cells:

1. Select the desired cell components to segment: Nuclei, Cytoplasm, and Membrane. Cytoplasm and Membrane can only be selected after Nuclei is selected.
2. Select **Discard if Touching an Edge** to discard any cell where any cell component touches the edge of the image, process region, or tissue region, if applicable. Only the cells that are entirely within the image, a process region, if applicable, and the selected tissue category, if applicable, are included in the segmentation map. If not selected, cells are clipped at the edge of the image or region. If a cell is clipped at the edge of a process region or tissue category region, the size of the nucleus or cytoplasm inside the region may be smaller than the specified Minimum Size.
3. If Nuclei is selected, click the Nuclei tab and select the desired parameters. See [Segmenting Nuclei](#)^[59] for descriptions of the parameters.
4. If Cytoplasm is selected, click the Cytoplasm tab and select the desired parameters. See [Segmenting Cytoplasm](#)^[63] for descriptions of the parameters.
5. If Membrane is selected, click the Membrane tab and select the desired parameters. See [Segmenting Membrane](#)^[64] for descriptions of the parameters.
6. Click the **Segment Image** button to segment the current image using the selected parameters.
7. If desired, change the parameters to obtain the desired results.
8. Click the **Segment All** button to segment all of the images.

13.1 Segmenting Nuclei

At the top of the **Cell Segmentation Settings** panel, select **Nuclei** in the **Compartments to Segment** box. (You can also select **Cytoplasm** and **Membrane** if you intend to find those as well.) Click the **Nuclei** tab.

– Selecting a Tissue Category

Select the **Tissue Category** in which to find the cell nuclei or select All Categories to find cell nuclei in all tissue categories. Cells that are outside the chosen category are ignored. This option only displays if tissue has been manually or automatically segmented.

Tissue categories of interest might be *tumor* regions in oncology samples, and *islet* cells in pancreas samples.

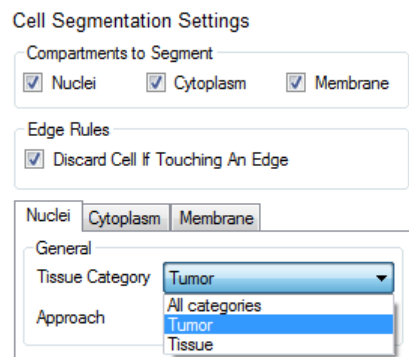


Figure 25. Cell Segmentation Settings for Nuclei

– Selecting an Approach

- Select the **Pixel-Based (Threshold)** approach when there is a reliable nuclear counterstain, and nuclear pixels can be found by applying a simple threshold. This approach is purely pixel-based. This approach can also be used for other image analysis needs that can be satisfied with a simple threshold, such as detecting all pixels within a tissue category that stain positive for an IHC stain.
- Select the **Object-Based** approach if the nuclear counterstain does not provide consistent and specific staining of nuclear objects, and more advanced morphometry-based approaches are needed to detect nuclei.

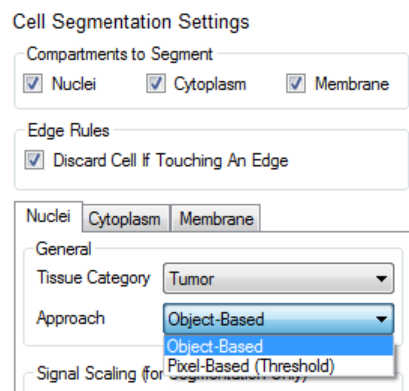


Figure 26. Cell Segmentation Approach

– Applying Signal Scaling

- **Auto Scale:** Select **Auto Scale** if you want the software to automatically scale each nuclear counterstain component plane individually before performing nuclei segmentation. This approach is a good place to start, and is useful if nuclear counterstain signals vary widely. Auto Scale is often sufficient for fluorescence applications.
- **Fixed Scale:** If you need better nuclear segmentation performance, and counterstain signals are consistent and reliable, you might get better results by selecting the **Fixed Scale** option. (The units are in parentheses.) This requires entering a scaling value, which can be determined by

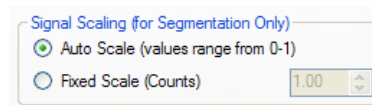


Figure 27. Signal Scaling

choosing the **View Component Data** tool from the toolbar and "mousing around" on the brightest nuclei to see the signal levels of the component being used for nuclear segmentation. Everything in a nuclear segmentation (indicated by a green region) should be nuclei.

- Reduce the maximum **OD** or **Counts** value if nuclear segmentations are smaller than the nuclei, or if no nuclear segmentations occur when the segmenter is finished.
- Increase the scale if segmentation is too sensitive and is finding many objects that are weakly stained and not nuclei. Adjust the scale until the unwanted regions are not detected.

— Selecting Components for Nuclear Segmentation

You can further add to what you consider nuclear pixels by including other components in the underlying signal. Pick an individual component signal as the primary, and then select secondary and tertiary components if desired. Enter a minimum signal value for each component. In **Auto Scale** mode, the signal range for each component is 0 to 1. If you selected the **Fixed Scale** option, the range for each component is from 0 to 2 for OD images, 0 to 255 for 8-bit images, and 0 to the Fixed Scale value for all other images. Also, whenever you switch between auto scale and fixed scale, the values are reset to zero.

Only pixels above the minimum signal value are counted as nuclei. Using secondary and tertiary components is primarily useful in brightfield IHC applications where nuclear IHC stains are dark or "intense" and substantially block the Hematoxylin counterstain. In these cases, performing nuclear segmentation on the logical intersection of the Hematoxylin and nuclear IHC signals is more effective. This approach does not work if the dark IHC stains reside outside the nucleus.

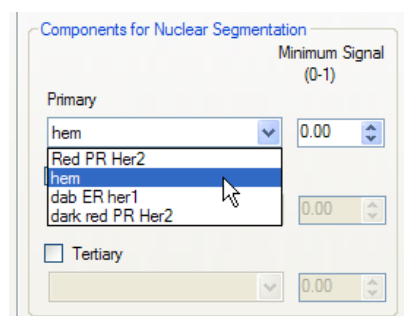


Figure 28. Components for Nuclear Segmentation

— Specifying a Nuclei Size Range

Set the **Minimum Size**. If too many small, unwanted structures (e.g., not nuclei) are found during segmentation, try increasing the minimum size value. Any nuclei that have fewer pixels than this number are not segmented, and no associated data is collected. If a nucleus is clipped at the edge of a Process region or Tissue Category region, the original size of the nucleus is used to determine if it meets the minimum size threshold; the clipped size is reported in the tables. For images taken at 20x magnification, 100 is a good starting value.

If appropriate, set the **Maximum Size**. This is required if you intend to select the **Refine Splitting** clean-up option discussed below. This sets an upper limit on nuclear

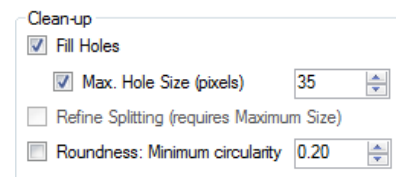


Figure 29. Size Range

segmentations (in number of pixels). Nuclear segmentations larger than this number are ignored. This can be useful if you want to ignore large clusters of nuclei, or if there are other objects in the classified region of interest that are not nuclei.

— Entering Nuclei Clean-up Settings

- Select **Fill Holes** when some nuclei end up segmented with “holes” in them. This results from a common effect in tissue sections where nuclei appear hollow. Checking this check box automatically fills holes in nuclei.
- If you want only those nuclei holes that are smaller than a certain size to be filled in, select **Max. Hole Size (pixels)** and enter the maximum number of pixels. Nuclei with holes that are larger than this value are not filled.
- If you select **Refine Splitting**, nuclei that are touching each other are split into separate nuclei instead of being detected as one large nucleus. This option is available only if **Maximum Size** is selected. Cell segmentation may take considerably longer when using this function. If you do not select **Refine Splitting**, nuclei that touch are detected as a single nucleus.
- Select **Roundness: Minimum circularity** to find only nuclei that are more round. Zero returns any shape. Higher decimal values (up to 1) restrict selections to more round objects only.



Clean-up

☒ Fill Holes

☒ Max. Hole Size (pixels) 35

☐ Refine Splitting (requires Maximum Size)

☐ Roundness: Minimum circularity 0.20

Figure 30. Clean-up options

13.2 Segmenting Cytoplasm

At the top of the **Cell Segmentation Settings** panel, select the **Cytoplasm** check box in the **Compartments to Segment** box. (Note that you must segment the nuclei in addition to segmenting cytoplasm. inForm only searches for cytoplasm where nuclei have been found.) Click the **Cytoplasm** tab.

— Selecting Cytoplasm Shape Parameters

- **Inner distance to nucleus:** The distance (in pixels) from the edge of nuclear segmentations to the inner edge of the cytoplasm annular regions drawn around nuclei. This buffer distance should be large enough to ensure that imperfections in the nuclear segmentation do not lead to signal from nuclei inadvertently becoming part of cytoplasm signals.
- **Outer distance to nucleus:** The distance (in pixels) from the edge of the nuclear segmentation to the outer boundary of cytoplasm segmentation. This distance should be adjusted based on visual inspection of the results, so that cytoplasm segmentation approximately matches the average size of cells. This distance can be set large enough to include membrane signals if desired. In this case, it is important to note that the signal extracted from the segmenter for cytoplasm is a mixture of membrane and cytoplasm signal.
- **Minimum Size:** The minimum cytoplasm sample size (in pixels). If cytoplasm segmentations for detected nuclei have fewer pixels than this number, those cells are excluded from the analysis. This is common in cases where cells are tightly packed together without much space between nuclei for cytoplasm areas to be segmented. If the cytoplasm is clipped at the edge of a Process region or Tissue Category region, only the cytoplasm inside the region is used to determine the size of the cytoplasm.

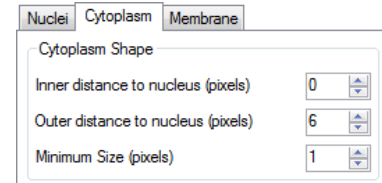


Figure 31. Cell Segmentation Settings for Cytoplasm

— Selecting Components to Include

You can further restrict what you consider “valid” cytoplasm based on the underlying signal. You can pick an individual component signal as the primary and then select secondary and tertiary components if desired, and enter a signal range for each one. Or select “*Every Component*” or “*Any component*” and specify a signal range. Only pixels that fall within that signal range are counted as cytoplasm. (The signal range varies based on image type.)

Component	Valid Signal Range		OD
	Min	Max	
<input checked="" type="checkbox"/> Primary			
Her2 - Fast Red	0.000	2.000	
<input type="checkbox"/> Secondary			
	0.000	2.000	
<input type="checkbox"/> Tertiary			
	0.000	2.000	

Figure 32. Cytoplasm Pixel Validation

13.3 Segmenting Membrane

At the top of the **Cell Segmentation Settings** panel, select the **Membrane** check box in the **Compartment to Segment** box. (Note that you must segment the nuclei in addition to segmenting membrane. inForm only searches for cytoplasm where nuclei have been found.) Click the **Membrane** tab.

— Selecting Components for Membrane Segmentation

1. Select the **Primary** component signal for membrane segmentation.
2. Select the **Secondary** component if desired.
3. For each component, set the **Full Scale**:
 - Select an image that is typical of all of the images to be analyzed.
 - Click the **Auto** button to find the brightest pixels in the image for each of the selected components. The brightest value for each component displays in the Full Scale box for each component. (The units can be counts or OD.)

Primary	Full Scale OD
ER - DAB	0.012
<input type="checkbox"/> Secondary	0.000

Auto

Figure 33. Components for Nuclear Segmentation

— Specifying the Segmentation Priority

The Segmentation Priority specifies which cell compartment (**Nucleus** or **Membrane**) has higher priority when segmenting a membrane that crosses a nucleus.

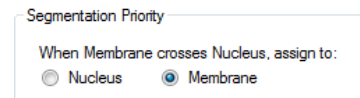


Figure 34. Segmentation Priority

— Specifying a Maximum Cell Size

The **Distance to Membrane** value specifies the maximum size of the cells, defined here as the distance (in pixels) from the edge of the nucleus to the outer edge of the membrane. A value of 12 for images taken at 20x magnification is usually a good starting value. If the algorithm is finding a membrane that is either too large or too small based on your knowledge of the nucleus/cell size, then try reducing (or increasing) the **Distance to Membrane** value. This value limits how far from the nucleus the algorithm is permitted to go when segmenting the cell membrane.



Figure 35. Maximum Cell Size

14 Scoring IHC or IF

After segmenting nuclei, cytoplasm, and membrane, the detected cell compartments are quantified. The options in the **Score IHC or IF Settings** panel provide functionality similar to that which is done manually or visually when pathologists assess chromogenic immunohistochemical stain levels. The Score IHC or IF settings specify the component thresholds and “score” the images.

1. **Tissue Category:** Select the tissue category to use for scoring. Cells that are outside the chosen tissue category are ignored. This option only displays if tissue has been segmented. Only tissue categories in which cells have been segmented are available.
2. **Scoring Type:** There are five Histogram score types, which are used to assess the intensity of each label. Select a score type from the **Scoring** box. Descriptions of each method are given below.
3. **Compartment:** Select whether you want to score the *Nuclei*, the *Cytoplasm*, or the *Membrane* cell compartment. If desired, you can score the nuclei, save the data, and then score the cytoplasm and/or membrane separately. The scoring data is a result of binning a histogram of all pixels within the selected compartment.
4. **Component:** Select the component to use for calculating scoring data.

Score IHC or IF Settings (research use only)

Tissue Category: Category1

Scoring: Positivity (2-bin)

Marker Settings

Compartment: ☒ Nuclei ☐ Cyto ☐ Membrane

Component: Hematoxylin

Threshold Max: 0.20 Auto

Positivity threshold (Optical Density)

0.0 0.2 0.032

Score Results (Percent)

☒ Show Score Colors

Change Color...

Negativity 56.27 % Positivity 43.73 %

Figure 36. Score IHC or IF Settings

5. **Threshold Max:** Click the **Auto** button to calculate the **Threshold Max** for the selected component. The Threshold Max specifies the maximum value of the **Threshold** sliders. If necessary, adjust this value up or down as needed. For OD converted images, 3.0 is the maximum possible value.
6. **Positivity Threshold:** Specifies the threshold values for the selected Scoring Type. Drag the slider, type the desired value in the text box, or click the up and down arrow buttons to change the Positivity Threshold value. See [Scoring Types](#)^[67] for descriptions of each Threshold.
7. **Show Score Colors:** If selected, the score colors display on the image and around the results percentage text boxes. If not selected, the colors do not display on the image or around the results percentage text boxes.
8. **Change Color:** Opens the Change Color window to select the colors for each bin or to restore the default colors to the bins.
9. When you score the image(s), the results display at the bottom of the Score IHC or IF settings panel. See [Scoring Types](#)^[67] for descriptions of the results for each scoring type.

Scoring Types

Positivity

This bins the spectrally unmixed nuclear, cytoplasm, or membrane signals into two bins, negative or positive, with a single threshold. (See Figure 30 above.) It provides data in percent. Decreasing the **Positivity Threshold** slider increases the percent positivity.

The **Score Results (Percent)** box shows the negativity and positivity percentages of the cell nuclei, cytoplasm, or membrane.

Double Positivity (2x2-bin)

Select this scoring method to classify cells, according to co-expression, into four classes: double negative, single positive for one marker, single positive for another marker, or double positive. The Negativity and Positivity percentages for each of the two markers are also shown.

Double Positivity contains the same options as Positivity with the addition of the following:

Second Marker Settings: Select the Compartment, Component, Threshold Max, and Positivity Threshold for the second marker after selecting the first marker settings.

Show Score Colors: Select the score colors to display on the image: Double Positivity, First Marker, or Second Marker.

To select the Double Positivity settings:

1. If the tissue has been manually or automatically segmented, select the **Tissue Category** as described above.
2. Select **Double Positivity (2 x 2-bin)** in the **Scoring** list box.
3. Under **First Marker Settings**, select the **Compartment** and **Component**, and then click the **Auto** button.
4. Under **Second Marker Settings**, select the **Compartment** and **Component**, and then click the **Auto** button.
5. To adjust the First Marker Threshold, select **First Marker** in the **Histogram Results** box and then adjust the First Marker Positivity slider or type the desired value in the Positivity text box. Positive regions are indicated by the Single Positive 1 color. Negative regions are indicated by the Double Negative color.
6. To adjust the Second Marker Threshold, select **Second Marker** in the **Histogram Results** box and then adjust the Second Marker Positivity slider or type the desired value

Score IHC or IF Settings (research use only)

Tissue Category:

Scoring:

First Marker Settings

Compartment: ☒ Nuclei ☐ Cyto ☐ Membrane

Component:

Threshold Max:

Positivity (Optical Density)

Negativity: **36.77 %** Positivity: **63.23 %**

Second Marker Settings

Compartment: ☒ Nuclei ☐ Cyto ☐ Membrane

Component:

Threshold Max:

Positivity (Optical Density)

Negativity: **100.00 %** Positivity: **0.00 %**

Histogram Results (Percent)

☒ Show Score Colors

Single Positive 1	Double Positive
63.23 %	0.00 %
Double Negative	Single Positive 2
36.77 %	0.00 %

Figure 37. Double Positivity Scoring

value in the Positivity text box. Positive regions are indicated by the Single Positive 2 color. Negative regions are indicated by the Double Negative color.

7. To view the double positivity colors, select **Double Positivity** in the **Histogram Results** box.

0-3+ (4-bin)

This option bins spectrally unmixed signals in nuclei, membrane or cytoplasm into four bins. This score type can be used to calculate H-scores with nuclear stains.

0/1 Threshold: Specifies the value below which to include cellular objects in the 0 to 1 bin.

1/2 Threshold: Cellular objects above the 0/1 threshold and below the 1/2 threshold are included in the 1 to 2 bin.

2/3 Threshold: Cellular objects above the 1/2 threshold and below the 2/3 threshold are included in the 2 to 3 bin. Cellular objects above this value are included in the 3+ bin.

Score Results (Percent): Shows the percentage positivity of the cell nuclei, membrane, or cytoplasm within each bin.

H-Score: The calculated H-Score of the image. The H-Score is calculated using the percentages in each bin and ranges from 0 to 300.

Score IHC or IF Settings (research use only)

Tissue Category: **Tumor**
Scoring: **0-3+ (4-bin)**

Marker Settings

Compartment: ☒ Nuclei ☐ Cyto ☐ Membrane

Component: **ER - DAB**

Threshold Max: **0.30** **Auto**

0 / 1 Threshold (Optical Density)

0.050
0.0 0.3

1 / 2 Threshold (Optical Density)

0.100

2 / 3 Threshold (Optical Density)

0.150

Score Results (Percent)

☒ Show Score Colors **Change Color...**

0 to 1 90.14 %	1+ to 2 7.21 %
2+ to 3 1.20 %	3+ 1.44 %

H-score

14

Figure 38. 0-3+ Scoring

Ten-bin

This histogram bins the data into ten bins for more quantitative work. The **10th Bin Nominal Level** slider specifies the ninth bin lower threshold. The other eight thresholds are equally spaced between zero and the ninth threshold.

The **Histogram Results** boxes display the percentages of pixels falling into the bins determined by the nominal level slider position.

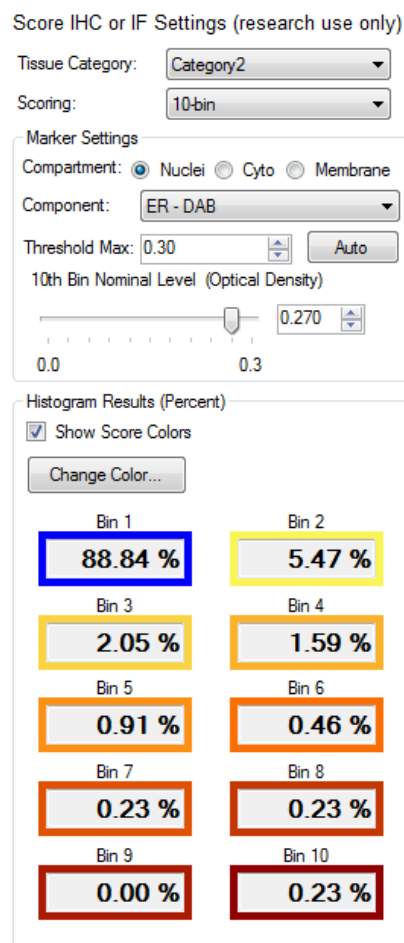


Figure 39. 10-bin Scoring

Fifty-bin

Divides signals into 50 equal bins.

The **50th Bin Nominal Level** slider specifies the lower threshold for the 50th bin. The remaining thresholds are equally spaced between zero and the 50th bin threshold.

For display, the 50 values are summed into 10 values, each value being the sum of 5 bins. The actual values for all 50 individual bins display in the Score table.

Score IHC or IF Settings (research use only)

Tissue Category: Category2

Scoring: 50-bin

Marker Settings

Compartment: ☒ Nuclei ☐ Cyto ☐ Membrane

Component: ER - DAB

Threshold Max: 0.30 Auto

50th Bin Nominal Level (Optical Density)

0.190
0.0 0.3

Histogram Results (Percent, groups of 5 bins)

☒ Show Score Colors

Change Color...

1-5	6-10
82.69 %	8.20 %
11-15	16-20
4.10 %	1.37 %
21-25	26-30
1.37 %	0.46 %
31-35	36-40
0.46 %	0.68 %
41-45	46-50
0.23 %	0.46 %

Figure 40. 50-bin Scoring

15 Counting Objects

Count Objects locates individual irregularly-shaped objects within an image field or within a selected tissue category if tissue has been segmented.

1. Click the **Count Objects** button in the step bar to display the **Object Counting Settings** panel.



2. If tissue has been segmented, select the **Tissue Category** in which you want to find the objects. Objects outside of the selected tissue category are not counted. (This option only displays if the project includes a Manual Classification or Segment Tissue step.)
3. Select the desired **Approach** to use to identify objects: **Object-Based** or **Pixel-Based (Threshold)**. Select the **Pixel-Based (Threshold)** approach when there is a reliable or consistent stain, and object pixels can be found by applying a simple threshold. This approach is purely pixel-based. Select the **Object-Based** approach if the stain does not provide consistent and specific staining of objects, and more advanced morphometry-based approaches are needed.
4. Select the desired **Signal Scaling**:
 - **Auto Scale**: Automatically scales each component plane individually before performing object segmentation.
 - **Fixed Scale**: If you need better segmentation performance, and stain signals are consistent and reliable, you might get better results by selecting the **Fixed Scale** option.
5. Select the **Primary** component for object segmentation from the drop-down list.
6. Adjust the **Minimum Signal** value for the primary component to the desired threshold value.
7. If desired, select **Secondary** and **Tertiary** components and minimum signal values.
8. To exclude objects smaller than a specific number of pixels, increase the value in the **Minimum Size (pixels)** text box.
9. To exclude objects larger than a specific number of pixels, select the **Maximum Size (pixels)** check box and specify the largest object size in pixels.
10. To automatically fill holes in objects, select **Fill Holes**. To limit the size of the holes that are automatically filled, select the **Max Hole Size** check box and specify the size in pixels of the

Object Counting Settings

General

Tissue Category
All categories

Approach
Object-Based

Signal Scaling (for Segmentation Only)

☒ Auto Scale (values range from 0-1)
☐ Fixed Scale (OD)

Components for Object Segmentation

Primary
Her2 - Fast Red
0.10

☐ Secondary
0.10

☐ Tertiary
0.10

Size Range

Minimum Size (pixels)
1

☐ Maximum Size (pixels)
10000

Clean-up

☒ Fill Holes

☐ Max. Hole Size (pixels)
0

☐ Refine Splitting (requires Maximum Size)
☐ Roundness: Minimum circularity
0.20

Edge Rules

☐ Discard Object If Touching An Edge

largest holes that should be filled.

11. To detect objects that touch other objects as individual objects, instead of as one object, select the **Refine Splitting** check box. The Maximum Size (pixels) check box must be selected to enable this option.
12. To exclude objects based on the roundness of the object, select the **Roundness** check box and specify the desired **Minimum Circularity**. Zero returns any shape. Higher decimal values (up to 1) restrict selections to more round objects only.
13. To exclude any objects that are touching the edge of the image, process region, or tissue region, select the **Discard Object if Touching an Edge** check box.
14. After processing, use the [View Editor](#)^[73] to view the resulting data tables. You can also export the Count Data table by advancing to the Export step ([Exporting the Data](#)^[89]).

16 Displaying the Extracted Data

After analyzing the images, use the [View Editor](#)^[19] to customize the view of the images and extracted data. These options do not change the underlying images, they only change how the images are displayed. The options below are available in the Data Displayed list (depending on which software configuration is installed and which steps are included in the project):

- [Viewing the Color Image](#)^[73]
- [Viewing the Component Image](#)^[74]
- [Viewing the Composite Image](#)^[77]
- [Viewing the Tissue Segmentation Data Table](#)^[78]
- [Viewing the Cell Segmentation Data Table](#)^[80]
- [Viewing the Score Data Table](#)^[82]
- [Viewing the Colocalization Data Table](#)^[84]
- [Viewing the Count Data Table](#)^[85]
- [Viewing the Quant Data Table](#)^[86]

16.1 Viewing the Color Image

Select **Color Image** in the Data Displayed list, then choose the desired options.

The following options are available for the Color Image:

Rendering Options

- **Brightness:** Change the value to change the brightness of the selected image. Lighten or darken the image using the Brightness percentage slider.
- **Contrast:** Change the value to change the contrast between light and dark portions of the selected image. Increase or decrease the contrast using the Contrast percentage slider.
- **Use Enhanced Contrast:** Select to artificially increase the vividness of the image.
- **Use Equal RGB Color Weighting:** (only for Fluorescence images) Select to distribute the wavelength range equally in the red, green, and blue components. If not selected, the image displays as it looks to the human eye.
- **Reset to Default button** - Click to reset the Rendering Options back to the default settings.

Image Options: See [Image Options](#)^[86].

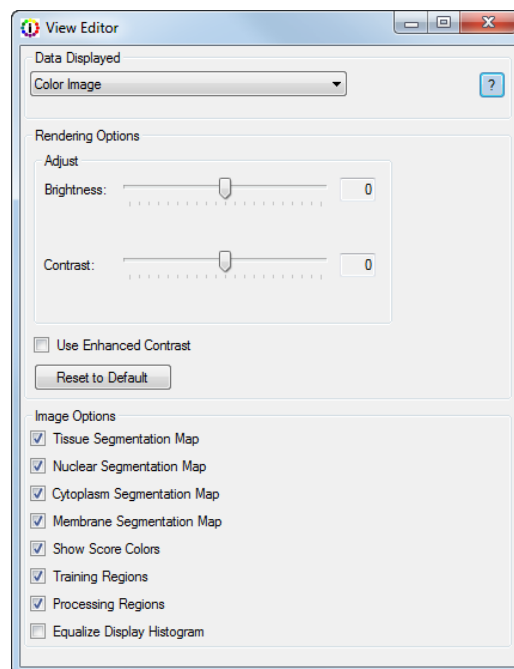


Figure 41. View Editor (Color Image)

16.2 Viewing the Component Image

Select a **Component** in the Data Displayed list, then choose the desired options.

The following options are available for the Component Image:

Rendering Options

Show As:

- **Brightfield:** Select to display the component signal on a white background.
- **Fluorescence:** Select to display the component signal on a black background.

Component Color:

- **Black and White:** Select to show the component as a black and white image. The component signal color is determined by the Brightfield/Fluorescence setting.
- **Color:** Select to show the component in color. This color is calculated from the spectral shape of the component, i.e. the color you would see when viewed through a microscope.

Scaling:

- **Scale Views for each Image Individually:** Select to show each image scaled individually. Dim images are brightened to show the components. The relative brightness of components cannot be compared between images.
- **Scale Views Equally for All Images in the Project:** Select to show the brightness of components for each image all scaled relative to each other. Bright components appear bright and dim components appear dim. All images are rescaled relative to all images in the project whenever images are removed from or added to the project.
- **Scale Views Based on Selected Images:** Select to show the brightness of components for each image based on the brightness range of the selected images. Bright components appear bright and dim components appear dim. The first time the option is selected, all current images in the project are selected. The scaling limits remain the same even if images are added to or removed from the project. Click the Reset button to open the [Scaling Image Selection Window](#)^[76] to change the images selected to determine the scaling limits. See [Scaling based on Selected Images](#)^[75] for the suggested workflow.

When this option is selected, the scaling limits are saved with the algorithm and project. If the algorithm or project is used in Batch Mode, then all images processed in the batch job use the saved scaling limits.

Brightness: Change the value to change the brightness of the selected image. Lighten or darken the image using the Brightness percentage slider.

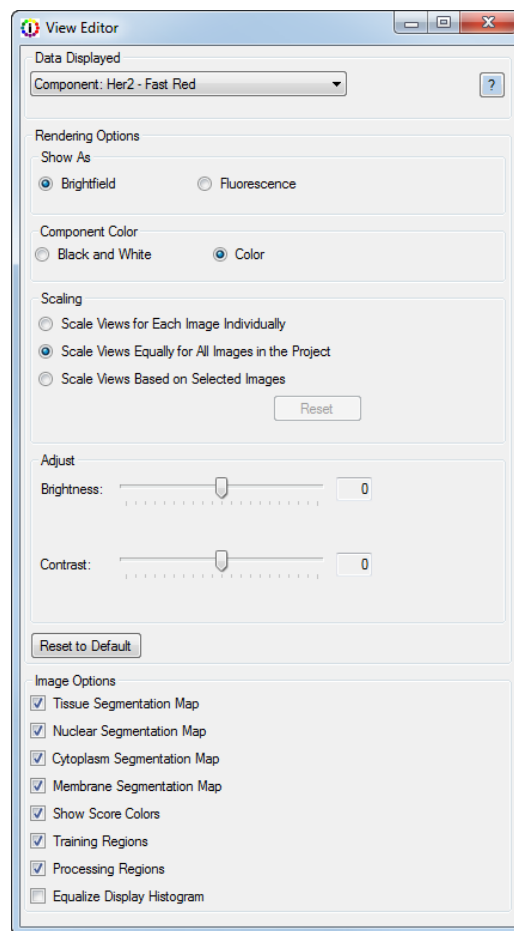


Figure 42. View Editor (Color Image)

Contrast: Change the value to change the contrast between light and dark portions of the selected image. Increase or decrease the contrast using the Contrast percentage slider.

Reset to Default button - Click to reset the Rendering Options back to the default settings.

Image Options: See [Image Options](#)^[86].

Scaling Based on Selected Images

Images with bright signals in each component, such as positive controls or positively expressing images, should be used to scale all images in the project.

When using Scaling Views based on Selected Images, the workflow below is suggested to set the desired scaling limits.

1. Load only the images of the positive controls for the images.
2. Process the images as desired.
3. Open the View Editor and select any component in the Data Displayed drop-down list.
4. Select the **Scale Views Based on Selected Images** option. The scaling limits are set based on the positive control images that are open in the project.
5. Save the algorithm and/or the project.
6. Use the settings for batch processing by loading the algorithm or project in the Batch tab.
7. If the positive controls change, open the project and click the **Reset** button on the View Editor. Select the new positive controls in the [Scaling Image Selection window](#)^[76] to set the new scaling limits.

Scaling Image Selection Window

Use the Scaling Image Selection Window to choose the images used to scale the brightness of all images in the project. The images that are brightest in at least one component are at the top of the list and are selected by default. Images must be unmixed to be used for the scaling calculations. If an image is not unmixed, it displays at the bottom of the list and the Select For Scaling check box is disabled.

Select For Scaling Check boxes: The scaling limits are calculated using all selected images. These scaling limits are saved and used when processing images in Batch Mode.

Use the Thumbnail and Image Name columns to identify the images in the project. The Image Name column also identifies images that are the brightest for each component.

Cancel button: Closes the Scaling Image Selection Window without recalculating any image scaling limits. The limits do not change.

Done button: Recalculates the image scaling limits using the selected images and then closes the window.

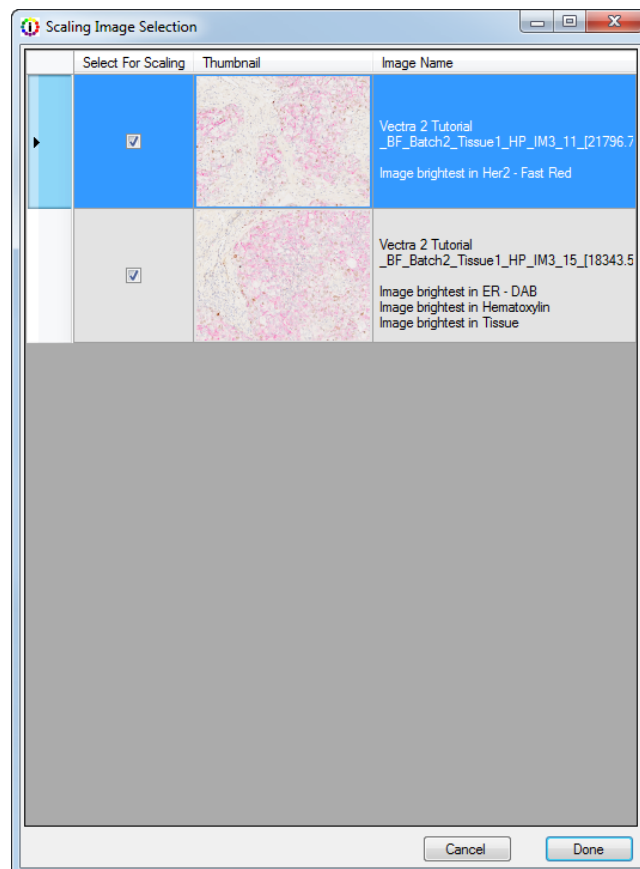


Figure 43. View Editor (Color Image)

16.3 Viewing the Composite Image

Select **Composite Image** in the Data Displayed list, then choose the desired options.

The following options are available for the Composite Image:

Rendering Options

- **Show As:**
 - **Brightfield:** Select to display the composite image on a white background.
 - **Fluorescence:** Select to display the composite image on a black background.
- **Scaling:**
 - **Scale Views for each Image Individually:** Select to show each image scaled individually. Dim images are brightened to show the components. The relative brightness of components cannot be compared between images.
 - **Scale Views Equally for All Images in the Project:** Select to show the brightness of components for each image all scaled relative to each other. Bright components appear bright and dim components appear dim. All images are rescaled relative to all images in the project whenever images are removed from or added to the project.
 - **Scale Views Based on Selected Images:** Select to show the brightness of components for each image all scaled relative to the selected images. Bright components appear bright and dim components appear dim. The first time the option is selected, all current images in the project are selected. The scaling limits remain the same even if images are added to or removed from the project. Click the Reset button to open the [Scaling Image Selection Window](#) ⁷⁶ to change the images selected to determine the scaling limits.
- **Display Intensity:** Select **True** to display each component in the composite image at the original intensity. For example, if Component A is dimmer than Component B, they display that way in the composite. Select **Adjustable** to display each component in the composite image at the intensity selected on the slider.
- **Display Color:** Select **True Color** to display each

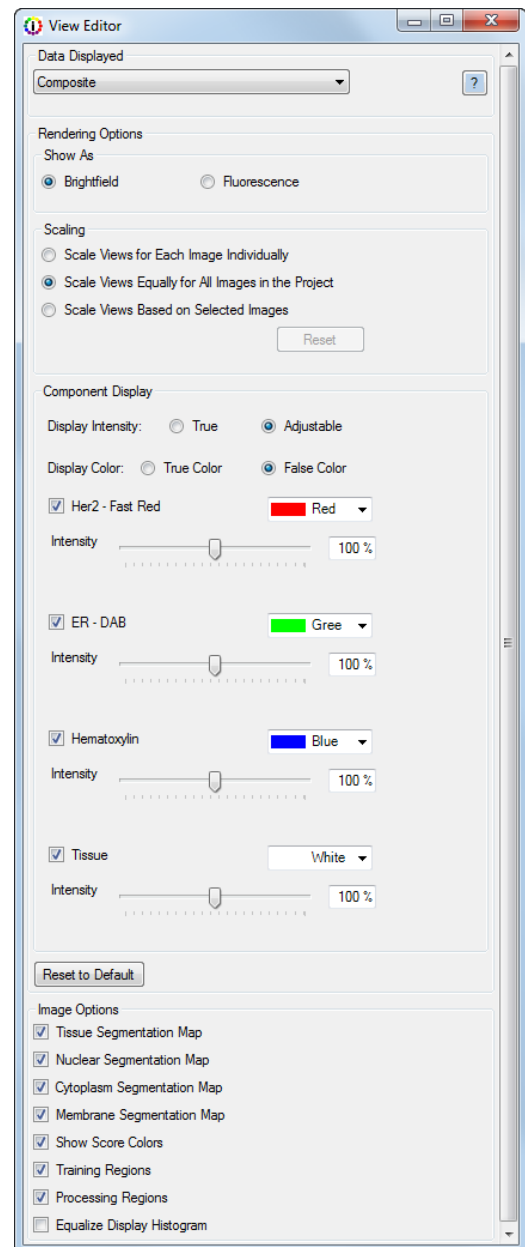



Figure 44. View Editor (Composite Image)

component in the calculated color from the spectral shape of the component. Select **False Color** to display each component in the selected false color. Select the False Color using the color picker next to each component (only visible when False Color is selected).

- **Component check boxes:** Select to display each component. If not selected, the component does not display. (The Intensity Slider for each component only displays if the Display Intensity is Adjustable. The Color Selector for each component only displays if the Display Color is False Color.)
- **Reset to Default button** - Click to reset the Rendering Options back to the default settings.

Image Options: See [Image Options](#) .

16.4 Viewing the Tissue Segmentation Data Table

The Tissue Segmentation Data Table is available when an algorithm contains a manual tissue segmentation or trainable tissue segmentation step. The table provides information on the manually or automatically segmented tissue regions. Select **Tissue Segmentation Data** in the Data Displayed list, then choose the desired options under **Table Contents**. Each option you select adds one or more data columns or rows to the table.

During image analysis, segmented tissue categories are assigned ID numbers. These numbers are used in the data tables to identify the tissue category for stats such as area (pixels), area (percent), etc.

The following options are available for the Tissue Segmentation Data Table:

Components: Select all or individual components for which to display component statistics. Each selected component creates one column for each component stat selected.

Tissue Categories: Select all or individual tissue categories for which to display data. An entry row is created for each Region ID in the tissue category.

Component Stats: Select the values to display for each component signal. See [Component Stats](#)^[87] for descriptions of each option.

Position Stats: Select which values to display for each segmented object. See [Position Stats](#)^[87] for descriptions of each option.

TMA Core Info: Select the options to include in the data table. See [TMA Core Info](#)^[87] for descriptions of each option.

Shape Stats: Select the options to include in the data table. See [Shape Stats](#)^[88] for descriptions of each option.

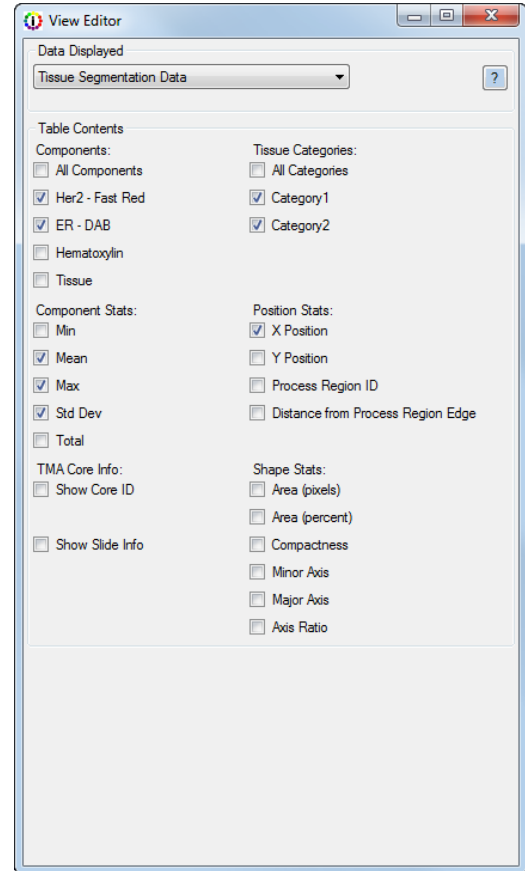


Figure 45. View Editor (Tissue Segmentation Data)

16.5 Viewing the Cell Segmentation Data Table

The Cell Segmentation Data Table is available when an algorithm contains a cell segmentation step. The table provides information on the segmented cells. Select **Cell Segmentation Data** in the Data Displayed list, then choose the desired options under **Table Contents**. Each option you select adds a data column or row to the table.

Cells are assigned sequential ID numbers according to the location of nuclei within each tissue category, if tissue has been segmented. ID numbering restarts at 1 in each tissue category. The table only shows statistics for cell compartments that have been segmented (nuclei, cytoplasm, membrane). For example, if cytoplasm is not segmented, the Cytoplasm column is not included in the table.

The following options are available for the Cell Segmentation Data Table:

Components: Select all or individual components for which to display component statistics. Each selected component creates one column for each component stat selected. If there is no data for a component, the component will not be displayed in the list.

Tissue Categories: Select all or individual tissue categories for which to display data. If selected, a Tissue Category column displays in the Cell Segmentation table to specify the Tissue Category for each cell. This selection does not display if there is only one Tissue Category defined or if there is no Segment Tissue step in the project.

Component Stats: Select the values to display for each component signal. See [Component Stats](#)^[87] for descriptions of each option. Each selection adds one column for each segmented cell component and one column for the entire cell.

- Cell statistics are calculated over the entire segmented area of the cell. Unsegmented areas are not included.
- The Entire Cell statistics are calculated using only the cell components that are segmented. (For example, if only nucleus and cytoplasm are segmented, the Entire Cell statistics will not include the membrane.)

TMA Core Info: Select the options to include in the data table. See [TMA Core Info](#)^[87] for descriptions of each option.

Position Stats: Select which values to display for each segmented object. See [Position Stats](#)^[87] for descriptions of each option.

Shape Stats: Select the options to include in the data table. See [Shape Stats](#)^[88] for descriptions of each option.

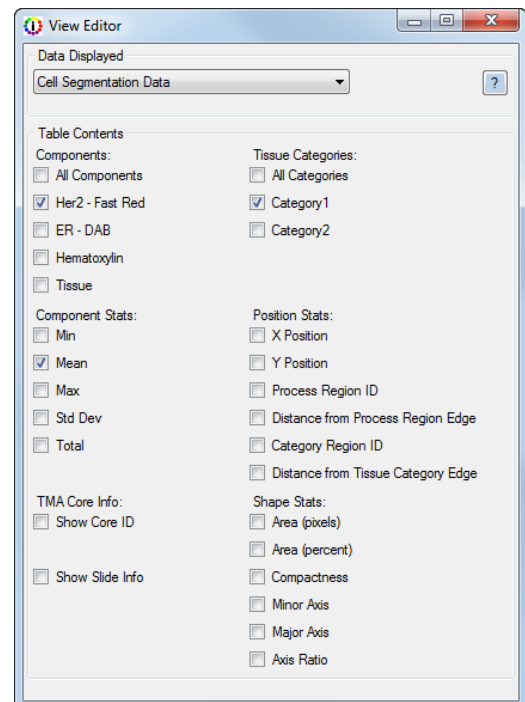


Figure 46. View Editor (Cell Segmentation Data)

The columns below are always visible in the Cell Segmentation Data table:

- **Cell ID:** Displays a unique ID number for each cell, based on the location of the nuclei within each tissue category, if tissue has been segmented.
- **Total Cells:** The total number of cells scored in the image.
- **Tissue Category Area (Pixels):** If tissue has been segmented (either automated or manual), the area of the selected tissue category, measured in number of pixels. If there is no tissue segmenting step, this column is the total number of pixels in the whole image.
- **Cell Density (per megapixel):** If tissue has been segmented (either automated or manual), displays the cell density for each tissue category: the number of cells in the tissue category area / (the number of pixels in the tissue category area / 1,000,000). If there is no tissue segmenting step, displays the cell density for the entire image: Total cells / (the number of pixels in the image / 1,000,000).

16.6 Viewing the Score Data Table

The Score Data Table is available when an algorithm contains a Score step. The available data changes depending on the type of scoring. Select **Score Data** in the Data Displayed list, then choose the desired options under **Table Contents**. Each option you select adds a data column to the table.

Table Contents: Select the options to include in the data table. See [TMA Core Info](#) ⁸⁷ for descriptions of each option.

The columns below are always visible in the Score Data table:

- **Tissue Category:** If tissue has been segmented (either automated or manual), the tissue category used for scoring displays in the table. If there is no tissue segmenting step, this column is blank.
- **Tissue Category Area (Percent):** If tissue has been segmented (either automated or manual), the area of the selected tissue category displays in the table. If there is no tissue segmenting step, this column is 100% (the whole image).
- **Number of cells:** The number of cells scored in the image.
- **(First) Cell Compartment:** The cell compartment used for scoring.
- **(First) Stain Component:** The stain used to create the threshold for scoring.

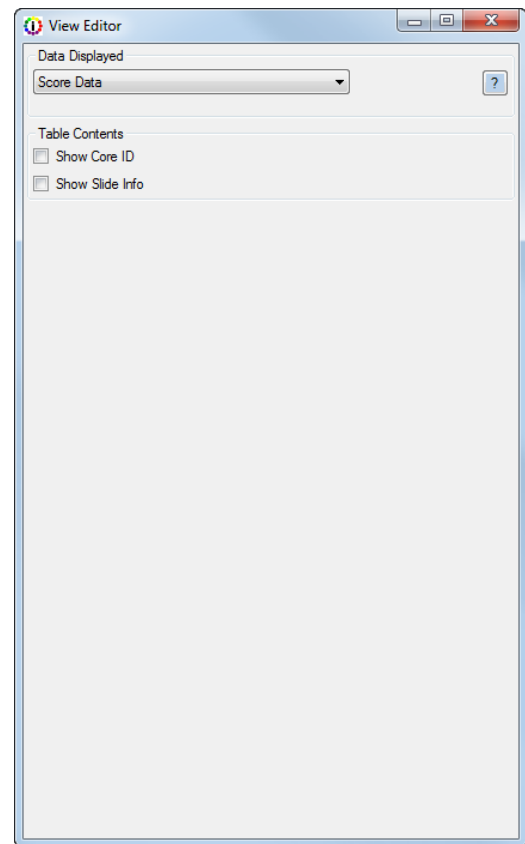


Figure 47. View Editor (Score Data)

The following additional columns are visible when **Positivity (2-bin) scoring** is selected:

- **Positivity:** The scored positivity.
- **Positivity Threshold:** The threshold value used to set the positivity.

The following additional columns are visible when **Double Positivity (2x2) scoring** is selected:

- **Second Cell Compartment:** The secondary cell compartment used for scoring
- **Second Stain Component:** The secondary stain used to create the threshold for scoring.
- **Double Negative:** The percent of cells that is negative (less than the threshold) for both components.
- **Single <Component1>:** The percent of cells that is positive for component 1 and negative for component 2.
- **Single <Component2>:** The percent of cells that is positive for component 2 and negative for

component 1.

- **Double Positive:** The percent of cells that is positive for both markers.
- **<Component1> Threshold:** The threshold used for component 1.
- **<Component2> Threshold:** The threshold used for component 2.

The following additional columns are visible when **0-3+ (4-bin) scoring** is selected:

- **0+:** The percentage of cells whose component strength is less than the first threshold.
- **1+:** The percentage of cells whose component strength is greater than or equal to the 0/1+ threshold and less than the 1/2+ threshold.
- **2+:** The percentage of cells whose component strength is greater than or equal to the 1/2+ threshold and less than the 2/3+ threshold.
- **3+:** The percentage of cells whose component strength is greater than or equal to the 2/3+ threshold.
- **H-score:** The calculated H-Score of the image. The H-Score is calculated using the percentages in each bin and ranges from 0 to 300.
- **Threshold 0/1+:** Clicking the Auto button sets this value to 1/4 of the Threshold max. This value can be changed.
- **Threshold 1+/2+:** Clicking the Auto button sets this value to 1/2 of the Threshold max. This value can be changed.
- **Threshold 2+/3+:** Clicking the Auto button sets this value to 3/4 of the Threshold max. This value can be changed.

The following additional columns are visible when **10-bin or 50 bin scoring** is selected:

- **Bin X (where X ranges from 1-10 or 1-50 per scoring type):** Columns showing the percent of scored values that fall in each scoring bin. The user sets the lower threshold for the last bin. The last bin is the percent of cells whose component strength is greater than the threshold the user set. The thresholds for the remaining bins are spaced evenly from 0. For example, the 49th bin has a lower limit of $(48 * \text{threshold}) / 49$.

16.7 Viewing the Colocalization Data Table

The Colocalization Data Tables are available when an algorithm contains a Colocalization step. Select **Colocalization Data** in the Data Displayed list, then choose the desired options for each table.

The following tables are included in the colocalization data:

Colocalization Table: Shows how much the numerator components are colocated with the denominator.

- **Percent Stats:** Shows the percent colocalization (where all colocalization pixels are positive within the selected denominator) and the percent positivity of each selected component in colocalization.
- **Pixel Stats:** Shows the actual pixel count for the colocalization, component positivities, and selected denominator.

In Channel Table: Shows the positivity of one component within another for all selected colocalization components.

- **Percent Stats:** Shows the percentage of inter-component positivity.
- **Pixel Stats:** Shows the pixel count of the component positivity.

Quant Table: Shows stats for the whole image and colocalization region.

Components: Select all or individual components for which to display component statistics. One column per numerator component is shown.

Component Stats: Select the values to display for each component signal. See [Component Stats](#)^[87] for descriptions of each option.

TMA Core Info: Select the options to include in the data table. See [TMA Core Info](#)^[87] for descriptions of each option.

Position Stats: Select which values to display for each segmented object. See [Position Stats](#)^[87] for descriptions of each option.

Shape Stats: Select the options to include in the data table. See [Shape Stats](#)^[88] for descriptions of each option.

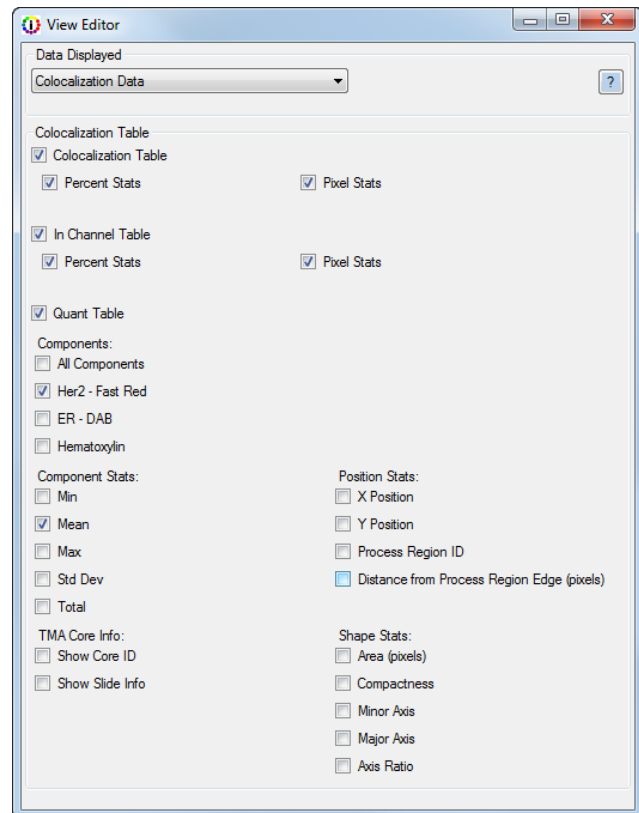


Figure 48. View Editor (Colocalization Data)

16.8 Viewing the Count Data Table

The Count Data Table is available when an algorithm contains an Object Segmentation step. Select **Count Data** in the Data Displayed list, then choose the desired options under **Table Contents**. Each option you select adds one or more data columns or rows to the table.

The following options are available for the Count Data Table:

Components: Select all or individual components for which to display component statistics. Each selected component creates one column for each component stat selected.

Tissue Categories: Select all or individual tissue categories for which to display data. If selected, a Tissue Category column displays in the Count Data table to specify the Tissue Category for each cell. This selection does not display if there is only one Tissue Category defined or if there is no Segment Tissue step in the project.

Component Stats: Select the values to display for each component signal. See [Component Stats](#)^[87] for descriptions of each option.

TMA Core Info: Select the options to include in the data table. See [TMA Core Info](#)^[87] for descriptions of each option.

Position Stats: Select which values to display for each segmented object. See [Position Stats](#)^[87] for descriptions of each option.

Shape Stats: Select the options to include in the data table. See [Shape Stats](#)^[88] for descriptions of each option.

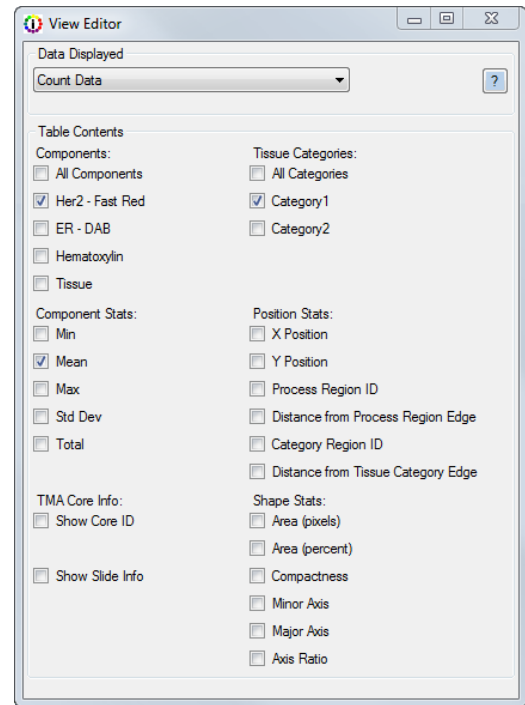


Figure 49. View Editor (Count Data)

16.9 Viewing the Quant Data Table

The Quant Data Table is available when an algorithm contains a Threshold or Colocalization step. Select **Quant Data** in the Data Displayed list, then choose the desired options under **Table Contents**. Each option you select adds one or more data columns or rows to the table.

The following options are available for the Quant Data Table:

Components: Select all or individual components for which to display component statistics. Each selected component creates one column for each component stat selected.

Component Stats: Select the values to display for each component signal. See [Component Stats](#)^[87] for descriptions of each option.

TMA Core Info: Select the options to include in the data table. See [TMA Core Info](#)^[87] for descriptions of each option.

Position Stats: Select which values to display for each segmented object. See [Position Stats](#)^[87] for descriptions of each option.

Shape Stats: Select the options to include in the data table. See [Shape Stats](#)^[88] for descriptions of each option.

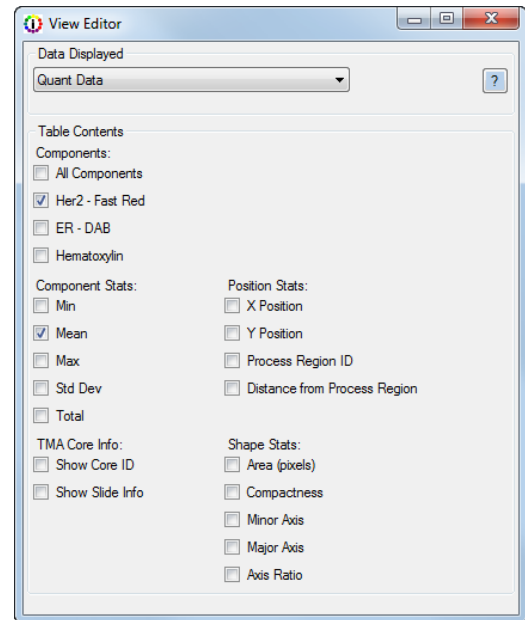


Figure 50. View Editor (Quant Data)

16.10 Image Options

The Image Options below are available on the [View Editor](#)^[19] when an image is selected in the **Data Displayed** list. Select or clear the check boxes described below to show or hide maps or regions on all images in the project. The buttons on the left side of the image display area also show or hide the maps or regions. Show Score Colors is also accessible on the Score Settings panel. The Equalize Display Histogram can only be shown or hidden here.

- Tissue Segmentation Map - Select to display the Tissue Segmentation Map on the image. (Only for projects that include a Manual Classification or Segment Tissue step.)
- Nuclear Segmentation Map - Select to display the Nuclear Segmentation Map on the image. (Only for projects that include a Segment Cells step.)
- Cytoplasm Segmentation Map - Select to display the Cytoplasm Segmentation Map on the image. (Only for projects that include a Segment Cells step.)
- Membrane Segmentation Map - Select to display the Membrane Segmentation Map on the image. (Only for projects that include a Segment Cells step.)
- Show Score Colors - Select to display the Score Colors Map on the image. (Only for projects that include a Score IHC or IF step.)
- Object Segmentation Map - Select to display the Object Segmentation Map on the image. (Only for projects that include a Count Objects step.)

- **Training Regions** - Select to display the Training Regions on the image. (Only for projects that include a Segment Tissue step.)
- **Processing Regions** - Select to display the Processing Regions on the image.
- **Equalize Display Histogram** - Select to map the pixels so the image histogram has approximately the same number of pixels assigned to each tonal value of the histogram. This gives the best display of the whole dynamic range of dim and bright signals. If not selected, the lowest 0.01% of the pixels are mapped to 0, the highest 0.01% are mapped to 255, and the remaining pixels are linearly interpolated between those values. This prevents a few bright or saturated pixels from skewing the display.

See [Displaying the Extracted Data](#)⁷³ for descriptions of other options available for each image type or data table.

16.11 Component Stats

The data tables contain the options below.

- **Min:** Displays one column containing the minimum value of the component in the region and one column displaying the .
- **Mean:** Displays the mean of the component in the region. The summary line displays the weighted mean value of the component across all regions, which is the average of the region means, weighted by region size.
- **Max:** Displays the maximum value of the component in the region.
- **Std Dev:** Displays the standard deviation of the values for the component in the region. The summary line displays the standard deviation of the individual means.
- **Total:** Displays the sum of the value of the component across all pixels in the region.

16.12 TMA Core Info

The data tables contain the options below.

- **Show Core ID:** If the image is of a core on a TMA slide scanned with a Vectra scanner, the Sector, Row, Column and Field of the core display in the table. If there is no core information, the columns contain zeros.
- **Show Slide Info:** If the image was taken with a Vectra scanner, the Lab ID and Slide ID from the file name display in the table. If there is no slide information, the columns are blank.

16.13 Position Stats

The data tables contain the options below. Units are in pixels.

- **X Position and Y Position:** The X and Y location of the center of the bounding box for the region.
- **Process Region ID:** If process regions are defined for the image, displays the ID number of the process region containing the tissue category. Displays #N/A if an object spans multiple process regions.
- **Distance from Process Region Edge (Pixels):** The distance, in pixels, from the center of this region's bounding box to the nearest edge of the containing process region. Displays #N/A if the

distance cannot be determined.

- **Category Region ID:** If tissue is segmented, displays the Region ID of the tissue category the segmented object is in. (Only available in the Cell Segmentation and Count Data Tables if tissue has been segmented.)
- **Distance from Tissue Category Edge (Pixels):** The distance, in pixels, from the segmented object to the nearest edge of the tissue category. Note that if the distance is reported as #N/A, this indicates that the distance is unknown or indeterminate, for example if a cell is in a tissue region that abuts the edge of the image. The cell may be closer to the edge of the image than to any other region in the image, so it cannot be determined whether the distance to an in-image point would be nearer than a point that lies beyond the edge of the image. (Only available in the Cell Segmentation and Count Data Tables if tissue has been segmented.)

16.14 Shape Stats

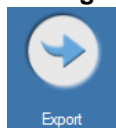
The data tables contain the options below.

- **Area (pixels):** The size, in pixels, of the region.
- **Area (percent):** The region size as a percent of the total processed area. If there are no processing regions, it is the total image area. Otherwise it is the total area of the processing regions.
- **Compactness:** The compactness of the region. Compactness is the ratio of the area of the region to the area of a circle (the most compact shape) having the same perimeter. A circle has a compactness of 1. Objects that are elongated or irregular are less compact, as are objects with irregular or reticulated edges.
- **Minor Axis:** The smaller of the two axes of the minimum area bounding box enclosing the region.
- **Major Axis:** The larger of the two axes of the minimum area bounding box enclosing the region.
- **Axis Ratio:** The ratio of the major axis / minor axis of the minimum area bounding box enclosing the region.

17 Exporting the Data

After analyzing the images, use the Export step to export images, component images, maps, and data tables. Images are exported in the format selected in the Export Settings step. Data tables are exported to tab-separated text files, which can be opened in Microsoft® Excel® and many other data analysis programs.

1. Click the **Export** button to display the **Export Settings** panel.



2. Click the **Browse** button to select or create the desired **Export Directory**.
3. Select the **Image Output Format** you want to export images to, either JPEG or TIFF. (Generally, TIFFs retain more resolution and are usually larger. JPEGs are compressed slightly and are often better for emailing and publishing on the web.)
4. From the **Images to Export** list, select the images and maps to export. Images can be exported with or without the maps. Exporting the images with maps can be used with Review/Merge to view the segmentation for all images in a batch.
5. Select the **Component Images (multi-image TIFF)** option to save a multi-image TIFF file of component data for analysis using third party analysis software. The values in the Component images are in the units selected in the Prepare Images step.

Components are saved in the spectral library order and the components are listed in order in the description tag of each of the component files in the stack.

6. Select the desired **Maps to Export**. Maps are exported alone, without the original image behind the map.

Note that the Cell Segmentation Map is a 'label' image that can be used in other image processing tools. Label images are 16-bit gray-scale TIFF images where all pixels from a segmented object have the same unique gray-scale value, up to 65,000 objects.

7. Select the desired **Tables to Export**. The tables available for export depend on which steps are included in the algorithm or project. Data tables are

Figure 51. Export Settings

exported to tab-separated text files, which can be opened in Microsoft® Excel® and many other data analysis programs.

8. In **Table Fields to Export**, select whether to export all available table data fields, or only the fields that are visible in the table view. To select which fields are visible in the table, use the **View Editor** (see [Displaying the Extracted Data](#))⁷³.
9. Select **File Name Options**: If files are already saved in the selected export directory, select how to name the new files. You can:
 - overwrite existing files if the new file names match, or
 - rename the new exported files to preserve any existing files in the export directory.
10. If you want to replicate the directory structure of the source images to the export directory, enter the number of directory levels to replicate in the **Copy __ level(s)** text box. For example, by entering "2" in this field, data in a "CancerStudy\Sample001" directory would be exported to the \<ExportDirectory>\CancerStudy\Sample001\ directory. This helps keep the exported images organized.
11. Click **Export For Selected** or **Export for All** to export the data.

The figure below shows a sample list of exported files.

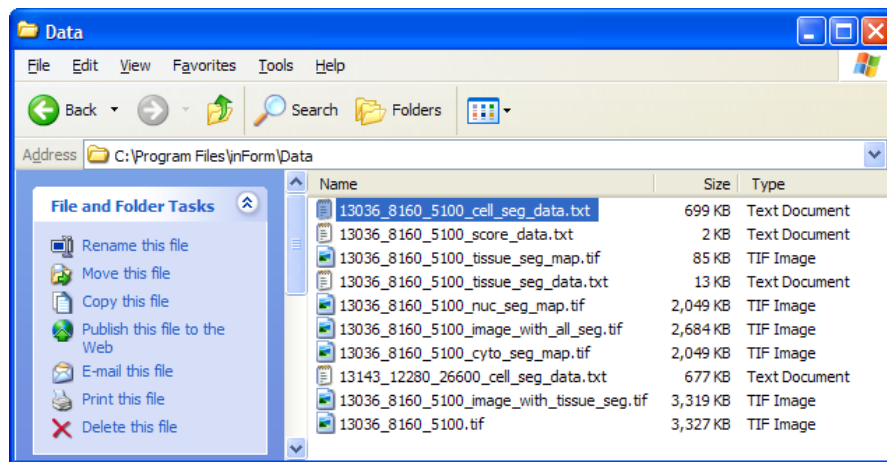


Figure 52. Exported Data Files

18 Batch Processing

Batch Analysis processes a large number of images quickly and effectively, with just one click of the batch **Run** button. Before beginning batch analysis, create and test an algorithm or project on a set of images that is representative of the entire data set. Adjust the algorithm or project as necessary until the images are processed as desired and then save the algorithm or project.

The screenshot shows the 'Batch Analysis' tab in the software interface. On the left is a vertical sidebar with three tabs: 'Manual Analysis', 'Batch Analysis' (which is selected), and 'Review / Merge'. The main area is divided into two sections. The left section contains configuration options: 'Batch Algorithm or Project' with a text field showing 'projects\SegTissueSegCellScore.tif' and a 'Browse...' button; 'Export Options' with a checkbox for 'Create separate directories for each image'; 'Export Directory' with a text field showing 'C:\Data' and a 'Browse...' button; 'Image export options' with a dropdown menu set to 'TIFF'; 'Images to export' with checkboxes for 'RGB', 'RGB with Tissue Segmentation Map', 'RGB with Cell Segmentation Map', 'RGB with Scoring Map', 'RGB with All Segmentation Maps', 'Composite Image', and 'Component Images (multi-image TIFF)'; 'Maps to export' with checkboxes for 'Tissue Segmentation Map' and 'Cell Segmentation Map (TIFF)'; 'Tables To Export' with checkboxes for 'Tissue Segmentation Data', 'Cell Segmentation Data', and 'Score Data'; 'Table Fields To Export' with radio buttons for 'Export all fields' (selected) and 'Use view settings'; and 'File Name Options' with a 'Copy' checkbox and a text field showing '0' followed by 'level(s) of directory structure from source image'. A 'Run' button is at the bottom of this section. The right section is titled 'Input Files:' and contains buttons for 'Add Images...', 'Add Slides...', 'Remove Selected', and 'Remove All'. Below these buttons is a list of input files: 'C:\Documents\Data\inForm\V2.0\Projects\Export1\Vectra 2 Tutorial _BF_Batch2_Tissu' and 'C:\Documents\Data\inForm\V2.0\Projects\Export1\Vectra 2 Tutorial _BF_Batch2_Tissu'.

Figure 53. Batch Analysis Tab

1. Click the **Batch Analysis** tab to configure the batch settings and select images for processing. You are prompted to save the open project. Click Yes to save the open project or No to close without saving.
2. Algorithms that contain the Manual Classification step are not compatible with batch processing. If the open project contains this step, the software will post a warning. Open a

project that does not contain steps that require user interaction, and then click the Batch tab.

3. Select a **Batch Algorithm or Project**. When you first switch to the **Batch** panel, the **Algorithm** settings are copied from the active project. You can run a batch using these settings, or click the Browse button to select an Algorithm (.ifp) or Project (.ifr) file that contains the desired settings. When loading a project, only the algorithm settings are used. The images from the project are not loaded or processed.
4. If desired, select the **Create separate directories for each item/image** option to save the exported files in a separate folder for each of the images or slides in the data set. This is recommended for large batch processes that result in many data files and images.
5. Select the desired export settings to export images, component images, maps, and data tables. Export settings are explained in detail in [Exporting the Data](#)⁸⁹.
6. Click the **Add Images** button to select images to process or click the **Add Slides** button to select single slides or folders containing multiple slides. A slide is a scan from the Vectra software. If a slide has been reviewed with Vectra Review, the batch run only includes the accepted fields. If the slide has not been reviewed, all fields are included in the batch run.
7. If necessary, use the **Remove Selected** button to remove selected images or slides from the batch or use the **Remove All** button to remove all of the images or slides from the batch.
8. Click the **Run** button. inForm processes the items one at a time and exports the specified data.
9. When the image processing is complete, click the **Done** button in the Batch Progress window.

19 Merging the Data

Use the Review/Merge tab to combine exported data files from multiple images or slides. Each type of data file from a batch run can be merged into a single file. For example, all Cell Segmentation data files can be merged into a single file that contains the Cell Segmentation data from all the images in the batch.

1. After running a batch, click the **Review/Merge** Tab.
2. The **Batch directory** defaults to the directory of the current batch process, or the last directory used for batch processing. Click the **Browse** button to choose a different directory of images/slides and data.
3. The data sets that can be merged display in the list. All data sets are selected by default.
4. Review the list of data sets and select the data sets to be reviewed:
 - Select or clear the check marks in the list to include or exclude specific data sets.
 - Click the **Include All** button to select all data sets in the list.
 - Click the **Include None** button to clear the check boxes for all data sets in the list.
5. When a data set name is selected in the list, thumbnails of the exported images for each data set display at the bottom of the window. Click on a thumbnail to view the image in the large image display area.
6. Review the images and accept or reject each data set:
 - Use the left or right arrow buttons next to the **Gallery** thumbnails to scroll through the exported images for each data set.
 - Click the **Yes** button to include the data set or click the **No** button to exclude the data set.
 - If desired, use the Merge hot keys listed on the bottom left of the window to review the exported images for each data set.
7. When all data sets have been reviewed, click the **Merge** button. Type a file name prefix to identify the new files as the merged files. The merged data files are saved as tab separated text files in the batch directory with the specified file name prefix followed by the name of the data table. Data from any excluded data sets are included in the <prefix>_rejected_... files.

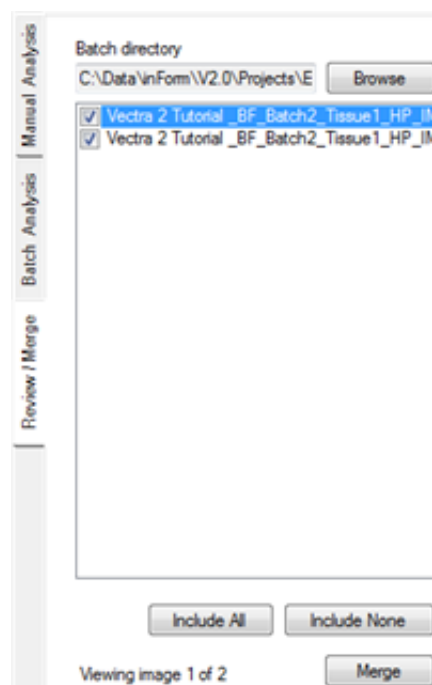


Figure 54. Review/Merge Tab

20 Creating Algorithms for Vectra

Customers who have purchased PerkinElmer's Vectra Intelligent Slide Analysis System use inForm to create Tissue Finder and High Powered Field (HPF) Finder algorithms for use with Vectra slide scanning protocols. The Tissue Finder algorithm differentiates between tissue and non-tissue regions. The HPF Finder algorithm finds specific targets such as tumor cells, inflammation, arterial plaque, etc. To fully utilize the imaging and tissue classification capabilities of the Vectra system, both of these algorithms need to be created for each unique set of tissue slides, and paired with their corresponding Vectra protocols.

— Selecting and Scanning a Slide Set for Algorithm Training

Before creating algorithms for a Vectra slide scanning protocol, use the Vectra Slide Analysis System to scan some slides selected from the slide set. Select enough sample slides so that heterogeneity of the entire slide set is represented in the sample slides. The sample slides can contain up to 20 slides, and should contain examples of all of the tissue types the algorithms will encounter when classifying images during the Vectra slide scanning protocol. You may want to scan two sets of sample slides; the first set can be used to train the algorithm and the second set can be used to test the algorithm.

In Vectra, load and scan the sample tissue slides manually, one at a time. Refer to the *Vectra User's Manual* for detailed instructions.

— Selecting Images for Algorithm Training

When you have finished scanning the sample slides, review the monochrome and color image data sets, and select images for algorithm training and testing as follows:

1. Select from 3 to 10 monochrome images (from the "Monochrome\fullres" Vectra directory). These images should contain examples of all of the tissue types that the algorithm will encounter. Group images that have similar phenomena into pairs and divide each pair into two separate folders. Images from one folder will be used for training the algorithm. Images from the second folder will be used to test the new algorithm.
2. Review the color image data set and select from 3 to 10 color images (from the "LPF\fullres" Vectra directory) representing all phenomena the algorithm might encounter. Again, group these images into two folders of up to 20 images each.
3. You can now use inForm to create the Tissue Finder and HPF Finder algorithms.

Note: While 3 to 10 images is usually sufficient, you may need more images to get a good segmentation of the target class across all slides if the target has wide variation.

— Creating Algorithms for Vectra

Create the Tissue Finder algorithm:

1. Choose **File > New > Project**. Choose the **Vectra Tissue Finder** option and click **Create**.

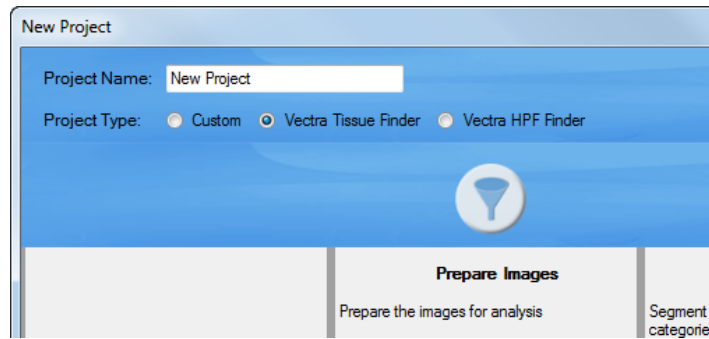


Figure 55. Vectra Tissue Finder project

2. Choose **File > Open > Image** and select the first group of monochrome images.
3. Click **Prepare All** to prepare the images.

Important! Do not perform a conversion of the images to optical density. Also, never change the resolution of the images for Tissue Finder or HPF Finder projects. **The algorithm will not work** with Vectra if the image resolution is changed.

4. Either click the **Advance** button at the bottom of the window or click the **Segment Tissue** button.
5. In the **Segment Tissue** step, two tissue categories are automatically created: **Target Tissue** and **Other**.
6. Draw training regions around tissue and around non-tissue regions on each of the images, using the appropriate tissue categories (see [Drawing Training Regions](#)^[48]).
7. Set the **Segmentation Options** as necessary for the current images (see [Training the Tissue Segmenter](#)^[49] for instructions). In most cases, you can select *Coarse* for the **Segmentation Resolution**. This allows the algorithm to classify images more quickly.
8. Click **Train Tissue Segmenter** to train the new Tissue Finder algorithm. When training is finished, segment the current image. If the segmentation looks good, segment all of the images.
9. To test and verify the Tissue Finder algorithm:
 - a. Click the **Available Images** button to open the [Available Images Window](#)^[21].
 - b. Remove the first set of images from the processing set, but leave them in the training set.
 - c. Load and segment the second set of images using the same algorithm.
 - d. Click **Segment All** and examine the resulting tissue segmentation.
 - e. If the algorithm needs improving, draw additional training regions on the second set of images. Click **Train Tissue Segmenter** again, and then segment all of the images again.

10. When you are satisfied with the Tissue Finder algorithm, click the **Advance** button or the **Export** button.
11. In the **Export** step, click the **Browse** button to select an export directory for the segmented monochrome images. The remaining export options are automatically selected and cannot be changed.
12. Click **Export for All** to export the segmented monochrome images to the specified directory.
13. Select **File > Save > Algorithm** to save the new Tissue Finder algorithm in the **Algorithms** folder of the **VectraData** directory. (Saving it here makes it easier to find the algorithm from the Vectra software.) The file name should identify it as the Tissue Finder algorithm for the specific set of tissue slides.

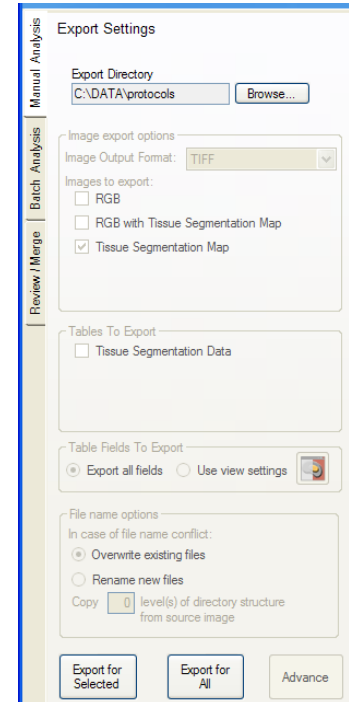


Figure 56. inForm Export Settings

Create the HPF (High Power Field) Training algorithm:

1. Create a new project, selecting the **Vectra HPF Finder** option in the [New Project Window](#)²².
2. Open the first set of low power RGB color images (from the "LPF\fullres" Vectra directory).
3. In the **Prepare Images** step, specify the sample format (brightfield or fluorescence).

Important! Do not perform a conversion of the images to optical density. Also, never change the resolution of the images for Tissue Finder or HPF Finder projects. **The algorithm will not work** with Vectra if the image resolution is changed.

4. In the Segment Tissue step, add the desired tissue categories, draw training regions, select the Segmentation Options, and train the Tissue Segmenter. Each of these steps is explained in detail in [Trainable Tissue Segmentation](#)⁴⁷.
5. When you are satisfied with the HPF Finder algorithm, export the images.
6. Select **File > Save > Algorithm** to save the new HPF Finder algorithm in the **Algorithms** folder of the **VectraData** directory. (Saving it here makes it easier to find the algorithm from the Vectra software.) The file name should identify it as the HPF Finder algorithm for the specific set of tissue slides.
7. The Tissue Finder and HPF Finder algorithms can now be selected in the Vectra tissue protocol.

21 Appendix A: Calculating Fractional Tissue Area

Fractional tissue areas can be calculated from inForm data using an Excel PivotTable. Examples of this type of calculation are:

- Tumor load: tumor area / (tumor + stroma)
- Lung fibrosis: fibrosis area / (normal lung + normal collagen + fibrosis)
- Breast tissue lobular involution: acini area / (acini + inflammation + lobular stroma)

It is also possible to calculate tissue fractions where the numerator and denominator are different tissue categories, such as:

- Kappa/lambda ratio in lymphoid tissue: kappa tissue area / lambda tissue area

This calculation can be performed per image or per slide with inForm (V2.0.1 or higher) and Vectra.

Initial Setup

These instructions assume that you already have an inForm algorithm capable of automatically segmenting the tissue categories of interest, and that you have processed several images using the algorithm and merged the tissue segmentation tables into a single table.

To compute tissue fraction by slide, you must have a column in the data table for slide ID. inForm includes this column for Vectra images.

The instructions in this section are for Excel 2010 and may need modification for other versions of Excel.

Create a PivotTable to Compute Fractional Tissue Area

To create the PivotTable:

1. Start Excel and open the data file. In the Open dialog, select "Text Files" in the file type drop-down, then select the inForm data file. The file name should end with "tissue_seg_data_summary.txt".
2. In the Text Import Wizard, if you are using a file from inForm V1.4, change the value for "Start import at row" to 3. This skips the first two rows. For inForm V2.0 or higher, leave the value at 1. Click "Finish" to open the file.
3. In the Insert ribbon, click "PivotTable". Leave the defaults: use the entire table and put the table on a new worksheet. Click OK to create the PivotTable. A new, empty PivotTable opens. On the right side, the PivotTable Field List shows all the fields in the source table.
4. Click the check box next to the name of the field to summarize by, usually Sample Name or Slide ID. It could also be TMA row and column. The selected field is shown in the Row Labels box and as a column in the pivot table.
5. In the PivotTable Field List, click the check box next to Tissue Category to add Tissue Category to the Row Labels list.
6. Drag "Tissue Category" from Row Labels to Column Labels.

7. Some tissue categories in the data may not be used in the tissue fraction calculation, for example a “Background” category. In this case, click the drop-down next to “Column Labels” and de-select any categories that are not of interest.
8. In the PivotTable Field List, click the check box next to Area (pixel) to add Area (pixel) to the Values box.
9. You should now have a PivotTable that shows total pixel area for each tissue category of interest and a Grand Total. The table has one row for each Sample Name or Slide ID, depending on the selection for Row Labels.
10. To display pixel area as a percent, right-click on “Grand Total” and select “Value Field Settings”. Select the tab “Show Values As”. In the drop-down, select “% of Row Total” and click “OK”.

You should now have a pivot table that displays percent of total area for each tissue category. The values of interest can be read directly from the table. For example, a “tumor” category displays the tumor load.

Compute a Ratio

The PivotTable does not easily display a ratio of two categories; it only shows each category as a fraction of the total. Additional ratios can be added as new computed columns outside the pivot table. Use explicit row/column identifiers in the formula, such as “D3”. Don’t use the `GETPIVOTDATA ()` function that Excel provides if you click on target cells. For example:

1. Click on the cell in the first empty column in the first data row.
2. Type the desired formula such as “=C5/B5” and press Enter.
3. Select the entire range in the target column, starting with the cell just entered.
4. Type CTRL+D to fill the column range with the desired formula.

Saving the Table

You can save the entire Excel workbook and/or export the PivotTable data as a text file for further processing with other programs. If exporting the PivotTable, you may want to refine the table for cleaner output. Below are some suggestions.

Remove the Grand Total Row at the Bottom

1. Open PivotTable Options by clicking the “Options” button at the left of the PivotTable Tools / Options ribbon.
2. In the Totals & Filters tab, deselect “Show grand totals for columns”.

Display Fractional Area

To display the tissue fraction as a decimal fraction rather than as a percent, click the drop-down next to the field name in the Values box. Select “Value Field Settings”. In the dialog box click “Number Format” and set the desired formatting.

Save as Tab-Separated Values

To save the file as tab-separated text:

1. In the **File** ribbon, choose **Save As**.
2. Select **Text (Tab delimited)** in the **Save as type** drop-down list. To save as a CSV file, select **CSV (Comma delimited)**.
3. Type the desired file name, select the desired location, and click **Save**.
4. Two warning messages display. First, "The selected file type does not support workbooks that contain multiple sheets." Click "OK". Then, "[FileName] may contain features that are not compatible with Text (Tab delimited)." Click "Yes".

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